Aspects of the haemostatic mechanism in newborns

ASPECTS OF THE HAEMOSTATIC MECHANISM IN NEWBORNS

Proefschrift

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Opgedragen aan
mijn moeder
Zij is, helaas, te vroeg
van ons heengegaan

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INTRODUCTION

Injury to the vascular endothelium is normally followed by haemostasis, i.e. the formation of a so called haemostatic plug. The formation of the haemostatic plug requires complex interactions between the vessel wall, blood platelets and a number of plasma proteins. Under normal conditions, these complex interactions are counterbalanced by a number of humoral and cellular protective mechanisms, in order to prevent an excessive reaction, which would lead to an impaired blood flow and the development of thrombosis.

The enzyme thrombin (Factor IIa) plays a key role in the formation of the haemostatic plug. Thrombin is formed from prothrombin, in a process katalysed by factor Xa, that, for all practical purposes, occurs only if both factors are bound to a negatively charged phospholipid surface. This binding occurs via extra negative charges (gammacarboxyglutamic acid (Gla) residues) which are build into the coagulation factors by a vitamin K-dependent mechanism. Thrombin formation is regulated by several positive and negative feedback mechanisms, which will be discussed below.

THE HAEMOSTATIC MECHANISM IN ADULTS

1. The role of the blood platelets

In vascular damage, the blood comes into contact with injured cells and a number of substances present in subendothelial tissue such as collagen, proteoglycans, fibronectin, elastin and microfibrils. Blood platelets adhere to these subendothelial structures (1). For a normal adhesion are required the von Willebrand factor complex, platelet membrane receptor proteins and fibronectin (1). The interaction between blood platelets and subendothelial structures activates the blood platelets. Activated blood platelets secrete a number of constituents, among them ADP, calcium and serotonin (from the dense-granules) and a number of platelet specific proteins, such as β-thromboglobulin, platelet factor 4 (heparin neutralizing factor), platelet growth factor and a number of proteins, which are also present in plasma, such as von Willebrand factor, fibrinogen, factor V, fibronectin and albumin. All these proteins are stored in and can be released from the α-granules (1). Activated platelets produce also the prostanoid thromboxane A_2, which is synthesized from its precursor arachidonic acid, derived from the platelet membrane. Thromboxane A_2 and ADP promote further aggregation and release of surrounding blood platelets. Thromboxane A_2 and serotonin have also vasoconstrictory properties (1). Simultaneously, the first traces of thrombin are formed via the activation of factor VII in the presence of tissue thromboplastin leaking from injured cells. (see below)

2. Thrombin-platelet interactions

If acting alone without additional stimuli, thrombin, even at a low concentration, will cause shape change, aggregation and the release reaction of platelets. The storage granules release their contents, including factor V. Traces of thrombin convert this factor V into its activated form. Apart from thrombin, many other substances can cause release, shape change and aggregation of the platelets. Of all substances (ADP, serotonin, collagen, adrenalin, thromboxane A_2, platelet activating factor (PAF), etc.), thrombin is the most active one on a molar basis. The concentration at which it shows half maximal activation is 10 to 1000 times lower than that of any other substance (except perhaps PAF). Among the activating substances, collagen holds a special position. In suspension, it will activate the platelet. In situations more akin to (patho-)physiological reality the platelets stick to collagen and spread on it, while being activated.

A crucial observation is that thrombin and collagen together will cause the outside of the intact platelet to become an active catalytic surface for the formation of thrombin (2). To explain the importance of this fact, we will first have to elaborate on the molecular biology of the clotting process.

3. The formation of thrombin

Clotting factors are proteins that occur in small quantities in the blood plasma. An exception is factor I, better known as fibrinogen which is present at 2-4 mg/ml. Factor II, present at 0.2 mg/ml, is usually called prothrombin, and the other factors are best known by their roman numeral designation. The vitamin K-dependent factors II, VII, IX and X are pro-enzymes, i.e. they become proteolytic enzymes after limited proteolysis just like trypsinogen becomes an active proteolytic enzyme trypsin after limited proteolysis e.g. by enterokinase. The activated forms of the clotting factors are indicated by the subscript a. Factor VIIa activates factor IX and X. Factor IXa activates factor X. Factor Xa activates prothrombin (factor II) to produce thrombin (factor IIa). In vascular damage, the blood comes into contact with injured cells and with collagen and microtubules in the connective tissue surrounding the vessel. From the injured cells leaks tissue thromboplastin (TTP), which consists of a phospholipid part and a protein part. Tissue thromboplastin (TTP) forms a complex with the (pro)enzyme factor VII, which binds to phospholipid surfaces via its gamma-carboxyglutamic acid (Gla) residues. This complex thus consists of two proteins, one of which is a serine protease, adsorbed together onto a phospholipid. Such a complex is up to 100.000 times more active than factor VIIa alone. In fact, the acceleration caused by tissue thromboplastin is so important that the reaction sequence is started when tissue thromboplastin becomes available. In the above, we have referred to both the pro-enzyme factor VII and the enzyme factor VIIa, which must seem illogical as only factor VIIa is the active enzyme. It appears, however,

that factor VII is an exception to the general rule in that it has a non neglectable enzymatic activity already in its pro-enzyme form. Also the product of the action of factor VII(a) on factor X, i.e. factor Xa, activates factor VII so as to form factor VIIa. In addition, the possibility exists that factor VII is activated by enzymes from injured cells, by factors from the contact activation system and by other proteolytic enzymes from the plasma.

The first small amount of proteolytic activity is thus easily obtained but it will only lead to full-blown thrombin generation if the subsequent amplifying mechanisms function properly. If this is not the case, the activity will be rapidly dampened by the antiproteases from the plasma (antithrombin III, α_2-macroglobulin, α_1-antitrypsin etc.). The first amplifying mechanism is formed by the reciprocal activation of factor VII by factor Xa. Factor Xa is the enzyme responsible for the activation of prothrombin. Just like factor VII(a), factor Xa needs phospholipids and an extra protein in order to attain full activity. The phospholipid and the accessory protein (also called helper protein or paraenzyme) in this case come from different sources. The accessory protein is factor Va that is formed from plasmatic factor V or from factor V released by the platelets by the action of thrombin. The phospholipid can be any negatively charged phospholipid, for example, phosphatidylserine.

TABLE I

The enzyme complexes

	enzyme	accessory factor	substrate	product
A	Xa	Va	II	thrombin
B	IXa	VIIIa	X	Xa
C	VII(a)	TTP	X, IX	Xa, IXa

Here we have to remark on a biochemical fact that is of the utmost importance in understanding the cooperation between platelets and clotting factors in the mechanism of haemostasis and the generation of thrombosis: all cell membranes, including those of the platelet, consist of a bilayer of phospholipids. This bilayer is asymmetrical, i.e. the phospholipids facing the surrounding medium have a composition that is, in part, different from the layer facing the inside of the cell.

The strong procoagulant phosphatidylserine is found entirely or almost entirely in the inner layer of the cell membrane. That is why intact cells do not show procoagulant activity. The phosphatidylserine present on the inside becomes available to the surrounding medium, only if cells lyse. To

this general rule there is one exception: the platelet. Like all other cells, the intact unactivated platelet has no (or minimal) procoagulant activities. Recently, however, it has been demonstrated, that a platelet, adherent to collagen and at the same moment exposed to thrombin, will present phosphatidylserine on its outer surface without losing its integrity. This is known as the transbilayer phospholipid transport or "flip-flop" mechanism. Knowing this, we can summarize the first phase of blood coagulation as follows:

Tissue damage exposes the blood to:

a. Tissue thromboplastin.

b. procoagulant phospholipids from the interior of injured cells

c. collagen and microfibrils from the connective tissue.

a and b together cause a small amount of factor X to be activated and, even if factor Va is absent, this factor Xa can activate some prothrombin in the presence of phospholipids. Even very small amounts of thrombin (the equivalent of less than 0.1% of the prothrombin present in a given volume of blood) will cause the platelets that adhere to the exposed collagen to activate their "flip-flop" mechanism. Also, they will release factor V which is activated by the thrombin present.

The second phase is characterized by the rapid formation of thrombin by factor Xa together with factor Va and platelet phospholipids, that in the vicinity of the site of injury are activated, for example, by ADP, prostanoids and ADP. This platelet aggregate is therefore a potent source of further thrombin formation.

The alternative pathway of activation of factor X by factor IXa we call the parallel phase of blood coagulation. Factor IXa requires factor VIIIa as an accessory protein and again a procoagulant phospholipid surface as is provided by stimulated platelets. If little tissue thromboplastin is available, the formation of factor Xa by factor VIIa is slow. In this case, the activation of factor IX by factor VIIa, in itself ten times slower then the activation of factor X, becomes important. This is evident if one realizes that the amount of factor IXa produced by factor VIIa determines the velocity of factor Xa formation by the parallel pathway. The amount of factor Xa thus generated will rise parabolically and, in the end, exceed the amount formed directly by factor VIIa.

If clotting times are long, e.g., in the case of diluted thromboplastins, the levels of factor VIII and IX become important for thrombin formation. In vivo, this cannot be denied a certain importance, as witnessed by the haemorrhagic tendency of the haemophiliac and by the fact that these patients tend to bleed in thromboplastin-poor tissues like joints and muscles.

The most important conclusions must be that blood coagulation and platelet reactions are so interwoven that it makes no sense to try to separate them. Thrombin is the most important activating agent for platelets and ensures their aggregation to form a stable aggregate. Blood platelets are an

indispensible source of phospholipids and also supply a part of the necessary factor V.

It will not have escaped the reader's attention, that we have not discussed the contact activation system. This system -Factor XII (=Hageman factor), Factor XI, prekallikrein (PK) and high molecular weight kininogen (HMWK) - is capable of activating factor IX and factor VII. It can be triggered by the contact of blood with glass or other negatively charged substances (kaolin) or, in vivo e.g. by antigen-antibody complexes. Its role in haemostasis and thrombosis is still unclear. It is worth noting that the contact activation system not only activates coagulation but also the fibrinolytic system, the kinin system and others. Deficiencies in the contact system will seldom lead to serious bleeding disorders. Serious bleedings in factor XI deficiencies do occur but have the character of being due to overactive fibrinolysis rather then to deficient clotting (U. Seligsohn, personal communication). In this context, it is an interesting piece of anecdotical evidence that Mr. Hageman (the patient in whom factor XII deficiency was first discovered) died of pulmonary embolism.

Before the activation of factor IX by factor VII(a) had been discovered, it was thought that only factor XIa was capable of activating factor IX. Following this line of thought, the contact activation system is compulsory in explaining the role of the antihaemophilic factors (IX and VIII). This activation, known as the intrinsic pathway (XII,XI,IX,X) was regarded as the alternative to the extrinsic pathway, operative in the presence of tissue thromboplastin (VII,X). We doubt the usefulness of this distinction for in vivo processes, although it may well describe the situation in the test tube, where tissue thromboplastin is either completely absent (partial thromboplastin time) or present in large excesses (prothrombin time). We prefer to use the reinforcement loop of figure 1 (called the "Josso loop" after the man who first described it) as the basis of a coagulation scheme. To complete this scheme we must include the accessory factors (tissue thromboplastin, factor VIII, factor V) and the phospholipids of the platelet membrane.

4. Fibrinogen and factor XIII

The final event during coagulation is the thrombin-induced transformation of the soluble fibrinogen into fibrin monomers, which spontaneously polymerize into an insoluble network. Factor XIIIa then mediates the stabilization of fibrin by crosslinking covalently neighbouring fibrin monomers. Plasma fibrinogen is a complex, dimeric glycoprotein with a molecular weight of 340.000. It is composed of three pairs of homologous polypeptide chains designated $A\alpha$, $B\beta$ and γ, held together by disulphide bridges (3). Carbohydrate is present on the β and γ chains, but its contribution to fibrinogen function is unclear (4). Fibrinogen is required for normal interaction of blood platelets with the vessel wall after injury (5). In vitro, the aggregation of washed human platelets by ADP is also

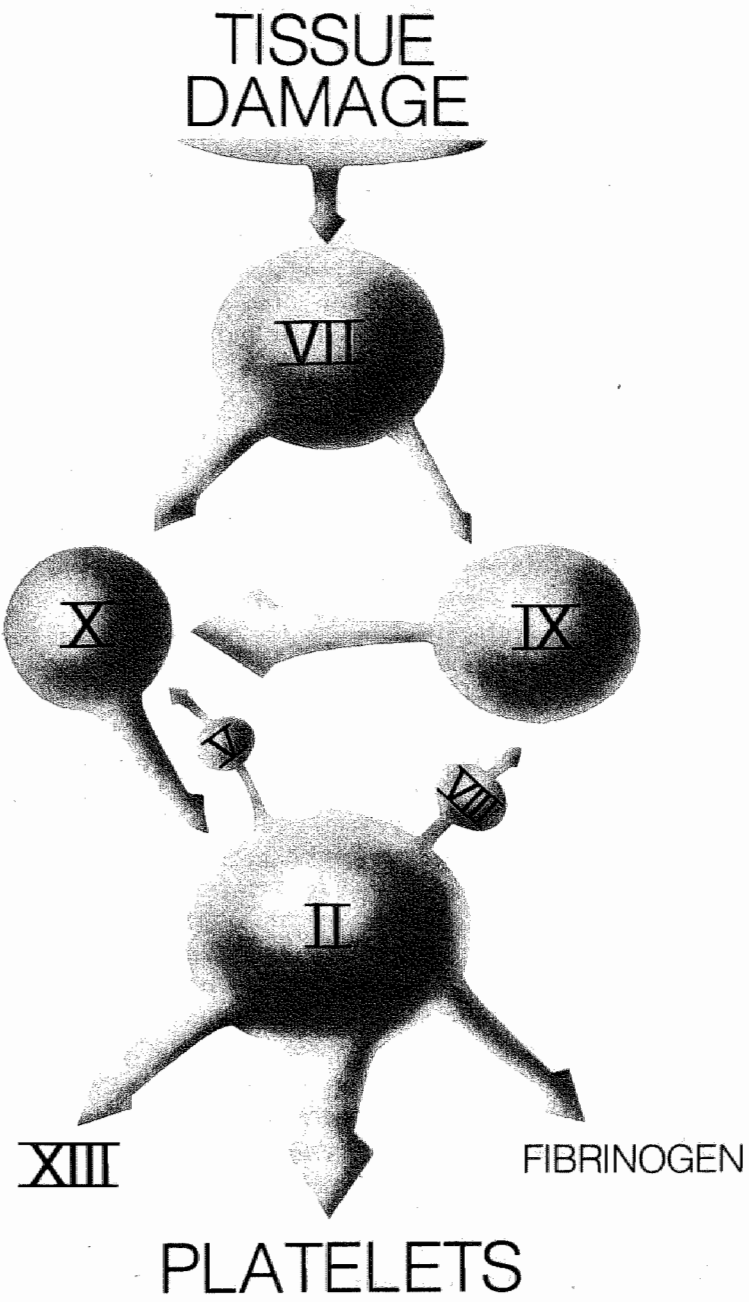


Figure 1: The basis of the coagulation scheme

dependent on the presence of fibrinogen (5). It has been shown that fibrinogen binds to specific receptors in the platelet membrane. This binding, requiring divalent ions, occurs in response to the ADP-induced platelet shape change (5). A binding site on fibrinogen, recognized by platelet receptors, is located in the γchain (5). Fibrinogen interacts also with some other proteins, notably with fibronectin (5). The binding site for fibronectin appears to be located in the carboxy-terminal portion of the fibrinogen Aα-chain (5). The negatively charged fibrinopeptides A (16 amino acids) and B (14 amino acids) represent the aminoterminals of the Aα and Bβ chain, respectively. Thrombin cleaves these fibrinopeptides off, which results in a change in charge distribution over the molecule and the exposure of two sets of polymerization sites, permitting the polymerization of fibrin into fibers and networks (3). The central domain, after the release of the fibrinopeptides, can bind to the terminal domain of unaltered fibrinogen. According to Doolittle's (1981) terminology of "knob and hole" interactions, the knobs generated by removal of fibrinopeptides A are able to interact with the ever present holes on the terminal domain of another molecule. This model allows for growth of a two-molecule-thick fibrin strand in both directions (6).
Plasma fibrinogen is synthesized in the liver. The α, β and γ-chains appear to be coded by separate genes. However, the translation of the three genes appears to be coordinated (7).

The importance of fibrin crosslinking by activated factor XIII became evident when in 1960 the first case of a patient with a factor XIII deficiency was described by Duckert (8). This patient suffered from haemorrhagic disease and impaired wound healing. Plasma factor XIII is a tetrameric molecule, composed of two a- and two b-chains, held together by non-covalent forces. The molecular weight of the tetramer is 320.000 (9). Activation of plasma factor XIII proceeds in several steps (10). Thrombin cleaves a small activation peptide from the a-chains. In the presence of calcium the b-chains dissociate and the active cystein residue becomes exposed. In the absence of fibrinogen unphysiologically high calcium-concentrations would be needed, but at normal fibrinogenconcentrations, physiological calciumconcentrations are sufficient. Factor XIIIa forms an amide bond between the γ-carboxyl group of glutamine and the ε-NH_2 groups of lysine residues. One consequence of fibrin crosslinking is increased mechanical strength of the clot.
Recently, it was shown that α_2-antiplasmin is also crosslinked to fibrin (11). This explains probably the relative resistance against plasmin digestion of clots formed in plasma. The fibrin α-chain can be crosslinked to fibronectin (12). Fibronectin has been shown to be crosslinked to collagen. This factor XIIIa mediated linkage of fibronectin to collagen could be important for tissue repair processes (13) and could explain the disturbed wound healing observed in factor XIII deficient patients (14).

5. Inhibitory mechanisms

Mechanisms that limit and localize the haemostatic process are critically important in order to protect against generalized thrombosis. In this section the actual view on the following inhibitory mechanisms will be discussed briefly:

A Anticoagulant properties of the vessel wall
B Blood flow and the reticulo-endothelial system
C Plasma serine protease inhibitors
D Protein, C, Protein S, APC inhibitor and thrombomodulin
E Feedback inhibitors
F Fibrinolysis

5A. Anticoagulant properties of the vessel wall

The inner surface of the intact normal vessel wall is non-thrombogenic. The luminal side of the endothelial cell-line is covered with a negatively charged glycocalix, which prevents adhesion and activation of blood platelets and coagulation factors. Another mechanism is the synthesis and release of prostacyclin, a potent vasodilator and inhibitor of platelet aggregation (15). Prostacyclin is formed from its precursor arachidonic acid via a number of intermediates.

Arachidonic acid is the most abundant fatty acid present in cellular phospholipids. Activation of phospholipase by a variety of changes in their chemical environment (15) results in the liberation of arachidonic acid, which is further metabolized by lipoxygenase and cyclooxygenase. Vascular prostacyclin synthesis occurs principally in the endothelium, whereas smooth muscle cells have considerable less prostacyclin synthetase activity. Endothelial cells can be stimulated to release prostacyclin by a variety of different agents, including thrombin, trypsin, ionophore A23187, bradykinin, interferon, histamine, high density lipoproteins and nitroglycerin (16,17,18,19,20,21). Damus et al (1973) suggested that heparin-like molecules on the endothelial surface might also contribute to the anticoagulant properties of the vessel wall (22). It was shown that heparin like molecules could be liberated from cultured endothelial cells (23) and capillary endothelium after in situ perfusion (24). In animal models, a heparin-like acceleration of thrombin and factor Xa inhibition by antithrombin III, provided by the endothelium, has been demonstrated (25).

That an endogenous heparin-like mechanism is also important in humans is indicated by the isolation of abnormal antithrombin III molecules, that function normally in the absence of heparin, but not in its presence, from patients with recurrent thrombosis (26, 27).

Marcum (28) suggested that heparin-like molecules on the endothelial surface might bind a small fraction of the plasma antithrombin III in vivo. That some antithrombin III is indeed bound to endothelium in life has been confirmed by Stern (29). Recently, a new anticoagulant protein has been described by Reutelingsperger (30). This protein was isolated from human

umbilical cord vessels and bovine aortae. This protein V.A.C. (vascular anticoagulant) is an inhibitor of the prothrombinase complex.
Another important contribution to the anticoagulant properties of the vessel wall is provided by the production of thrombomodulin, which acts as an endothelial cell protein cofactor for the thrombin-catalyzed activation of protein C. This will be discussed below.
Interestingly, thrombin complexed with purified thrombomodulin, loses its ability to clot fibrinogen, to activate factor V and to aggregate platelets (31,32). The endothelial cells are also involved in the fibrinolytic system by the production and release of plasminogen activator and an inhibitor of this activator (see section on fibrinolysis). In the literature a few data are available for an active role of the vessel wall in the uptake and degradation of procoagulant substances such as ADP (33) and vasoactive peptides (34), therefore also contributing to limiting the extent of the haemostatic reaction.

5B Blood flow and the reticulo-endothelial system

It must be recognized that, in vivo, coagulation does not occur in the closed stirred system generally used to study it in vitro. Blood flow through the wound area constantly brings fresh factors and platelets and washes away products that then as a rule are eliminated by the reticulo-endothelial system (R.E.S.), especially in the liver. The liver has the capacity to remove selectively activated clotting factors from plasma, without altering the concentration of the unactivated species (35).
The fact that most of the coagulation reactions occur at lipid interfaces tends to localize the site of the reactions. Other reactions, such as the thrombin-thrombomodulin-protein C interactions are localized at the endothelium surface near the wound. The actual flow conditions seem to be important in the generation of thrombosis. Their effect on haemostasis is as yet incompletely understood.
A fact to be reckoned with is that the high pressure gradients caused by the cutting of a vessel induce sudden increases of plasma flow velocity that tend to activate platelets by shear stress.

5C Plasma serine protease inhibitors

Several plasma protease inhibitors have been shown to inhibit the activity of activated coagulation enzymes: antithrombin III (AT III), C1-inhibitor (C1-inh), α_1-antitrypsin (α_1-AT), α_2-antiplasmin (α_2-AP), α_2-macroglobulin (α_2-M) and heparin cofactor II (HC II). Most inhibitors are able to neutralize several different proteases in vitro.
-Antithrombin III is a single chain glycoprotein (MW 58.000) with a normal plasma concentration of 100-150 µg/ml (36). AT III is considered to be the main physiological inhibitor of thrombin and factor Xa (37), plasma kallikrein (38) and plasmin (39) in purified systems. Heparin greatly enhances the reaction rate of the enzyme-AT III interaction. Heparin binds to AT III

(a short specific sequence of sulphated and non sulphated monosaccharide units) without changing the stoichiometry of the AT III-enzyme complex. This results in a conformational change of the inhibitor resulting in the acceleration of the inhibitory effect (40,41).
Heparin can dissociate from the complex and be reused in the reaction (42). It has been found that high affinity, low molecular weight heparin fractions have a relatively greater antifactor-Xa than anti-IIa effect, whereas high molecular weight fractions have a relatively higher antithrombin (IIa) than antifactor Xa activity (43). The physiological importance of antithrombin III is underlined by the tendency to recurrent venous thromboembolic disease in AT III deficient patients and in patients with abnormal antithrombin III molecules.

-C1-inhibitor is a single chain plasma glycoprotein (MW 105.000) containing a high proportion of carbohydrate (35%) (44). Despite the obvious importance of this inhibitor as a regulator of the contact activation phase, the congenital deficiency state (hereditary angioneurotic oedema) is not associated with any thrombotic or haemostatic disorder (25).

-α_1-Antitrypsin is a single-chain glycoprotein (MW 55.000) with a relatively high plasma concentration 2.5 mg/ml (45). It inhibits thrombin slow in vitro but does not contribute any significant part to the overall antithrombin activity of whole plasma (46). It contributes to 70% of the overall anti factor XIa activity in plasma (47). α_1-Antitrypsin appears not to be an important regulator of the coagulation system.

-α_2-Antiplasmin is a single chain glycoprotein (MW 70.000). The reaction rate between plasmin and α_2-AP is one of the fastest described for protein-protein interactions (48). It also inhibits β-factor XIIa, plasmakallikrein, factor XIa and thrombin (49) and factor Xa (50). The contribution of α_2-AP to the overall inhibitory effect of plasma against these enzymes, seems to be of minor importance. A hereditary deficiency of this inhibitor is associated with a severe bleeding tendency (80).

- α_2-Macroglobulin is a very large, tetrameric glycoprotein (MW 725.000) (51). α_2M has a wide spectrum of protease inhibitory potential, not limited to serine proteases (52). Among the coagulation proteases, only thrombin and plasma kallikrein have been demonstrated to the inhibited by α_2M in vitro. α_2M contributed 35-50% to the overall antikallikrein activity of plasma (53) and 25% of the overall plasma antithrombin activity (54).

-Heparin cofactor II is a new heparin dependent glycoprotein, that recently has been isolated from human plasma (55). HC II-thrombin interaction is accelerated nearly 1000 times in the presence of high heparin concentrations (55). These heparin concentrations are much higher than those usually administered to patients on heparin treatment for thromboembolic disease. The inhibition of Xa, however, was reported not to be accelerated by heparin. It also has been reported, that another mucopolysaccharide, dermatan sulphate, strongly accelerates the inhibition of thrombin by HC II but not by AT III (56). The in-vivo importance of HC II remains to be

established. Until now, hereditary HC II deficiency is not reported to be unconditionally associated with thrombotic disease. In a few case reports a possible relation was suggested (57,58).

5D Protein C, Protein S and activated protein C inhibitor (APC inhibitor)

The endogenous anticoagulant system involving the vitamin K-dependent proteins protein C (PC) and protein S (PS) has recently attracted much attention due to the discovery of a thrombotic tendency in patients with a PC deficiency (59,60) and PS deficiency (61,62).

PC is a vitamin K-dependent serine protease zymogen (MW 62.000, normal plasma concentration 4 µg/ml). PC must be activated to become an anticoagulant. Thrombin, the only known physiological activator removes a 12 residue peptide from the amino terminal end of the heavy chain, leaving the gla-containing light chain unaffected (63). The in vitro activation of PC to activated protein C (APC) by thrombin was shown to be dramatically increased when thrombin and PC were perfused through the microcirculation of the isolated rabbit heart (64). This led to the discovery and isolation of an endothelial cell protein cofactor: thrombomodulin (64). The enhancing effect required the presence of calcium. In addition human factor Va and Va light chain accelerate PC activation by thrombin without the need of calcium (65). Thrombomodulin is at least 20 times more efficient than human factor Va light chain (66).

It has been hypothesized that factor Va and thrombomodulin might synergistically enhance APC generation.

APC exerts its anticoagulant properties by proteolytically destroying factor V and factor VIII coagulant activities (67). The thrombin activated cofactors Va and VIIIa are destroyed more rapidly than the native pro cofactors. The degradation is greatly accelerated by phospholipids and calcium.

Another anticoagulant property of APC is its profibrinolytic activity, which is mediated via the inhibition of tissue plasminogen activator (tPA) inhibitor (68). Another vitamin K-dependent protein, protein S (PS) functions as a cofactor in the inactivation of factor Va, (69) and factor VIII and VIIIa (70). PS is able to bind calcium and in the presence of calcium it binds to phospholipids with higher affinity than other vitamin K-dependent proteins (71).

The APC induced prolongation of plasma clotting times is nearly abolished when plasma has been depleted of PS and can be restored by the addition of PS to plasma (69). It has been hypothesized that PS exerts an additional role in controlling complement activation (72). Thrombin cleavage of PS causes loss of the cofactor activity for the APC induced factor Va inactivation (73). It is unknown, if this reflects an in vivo regulatory mechanism. APC inhibitor has been purified by Suzuki (74), a single chain glycoprotein (MW 57.000, normal plasma concentration approximately 5 µg/ml). Assessment of the clinical significance of this inhibitor will await detection of deficient patients.

5E Feedback inhibitors

Thrombin generation also acts to abort the coagulation sequence. The effect of thrombin on factor V and VIII is biphasic. Initially cleavage by thrombin, increases the activity of factors V and VIII. Continued proteolysis by thrombin destroys the activity of factor V and VIII, so that they can no longer participate in thrombin formation. Thrombin is also able to cleave prothrombin fragment 1 from prothrombin, thus generating prethrombin 1, which lacks the gla-region and does not bind to phospholipids. The hypothesis that thrombin may regulate its own formation via this negative feed-back, has been questioned, because no corresponding fragments were found after blood clotting in vitro (75). Another feedback mechanism has been postulated by Rosenberg (36). Fibrinogen polymerization is inhibited when plasmin cleaves the Bβ chain at position 42-43 after thrombin-induced release of the fibrinopeptides A. Via this mechanism plasmin could serve as an anticoagulant besides its role in degrading fibrin.

5F The fibrinolytic system

Coïncident with the formation of a fibrin clot, an enzymatic system is initiated to remodel or remove the clot and restore the blood flow in the damaged vessel wall. Fibrinolysis is mediated by the enzyme plasmin (76) which is a relatively non-specific endopeptidase. It cleaves a variety of proteins such as fibrin(ogen), factor V and VIII, complement components, ACTH and growth hormone. Each of these proteins contains suspectible arginine and lysine bonds (76). Plasmin circulates in its inactive form plasminogen, which has a high binding affinity for fibrin as it polymerizes to form a clot.

Plasminogen can be activated to form plasmin by several plasminogen activators: the so called intrinsic activators (present in the circulating blood) and extrinsic activators (present in endothelial cells and in other tissues). In plasma a protein is present with affinity for the lysine-binding sites in plasminogen. Theoretically this "histidine-rich glycoprotein" may serve as an antifibrinolytic agent (77). The extrinsic activators can be released upon a variety of stimuli, especially the tissue type plasminogenactivator (tPA) derived from the endothelial cell binds strongly to fibrin, rendering it very active in the formation of plasmin from fibrin bound plasminogen. In endothelial cells also an inhibitor of tPA is synthesized (78). This inhibitor is probably also synthesized by human hepatocytes (79).

To protect the general circulation from effects of plasmin, plasmin liberated from the dissolving clot is rapidly neutralized by an inhibitor present in plasma, α_2-antiplasmin (48). A hereditary deficiency of this inhibitor is associated with a severe bleeding tendency (80).

A number of excellent reviews on the fibrinolytic system have been published. As a more detailed review of this system is beyond the scope of this thesis we refer the reader to Collen (76), Francis (81), Aoki (82), Bachmann (83) and Brommer (84).

THE HAEMOSTATIC MECHANISM IN NEWBORNS

Several key aspects of the haemostatic mechanism in newborns are defective when compared to adult standards (85). Quantitative as well as qualitative differences have been described. Tremendous variations occur in many of the components of the haemostatic mechanism, making the newborn infant prone to both haemorrhage and thrombosis (86). Most of the information currently available is based upon functional assays rather than biochemical or physiochemical techniques. In the following section the current data on the various components of the haemostatic mechanism in newborns are summarized.

1. Vessel wall

Small preterm newborns may bruise excessively after minimal trauma. Their predisposition to serious intraventricular haemorrhage has been in part explained by inadequate connective tissue support of capillaries and small venules (87). However, no hard data are available to support the clinical impression that the so called "vascular fragility" is indeed increased in these infants (88). The methodology of evaluating the in vivo vessel wall function is imprecise and poorly standardized. According to Buchanan (88) the vessel wall function in normal full term newborns is comparable with that of adults.

In the last decade, our knowledge of the properties of the vessel wall has rapidly grown, since it has become possible to culture endothelial cells (89). Many data in man, have been obtained by using endothelial cell cultures derived from umbilical veins. It remains to be established whether these data can be extrapolated to the adult situation.

2. Blood platelets

Platelets have been identified in fetuses of greater than 11 weeks gestation (90). Healthy newborn infants, even the smallest premature babies have platelet counts in the same range as normal adults. Therefore, a platelet count of less than 150×10^9/l indicates thrombocytopenia, regardless of the gestational age (91). There is some evidence that newborns have a mild transient defect in platelet function. A number of investigators have reported a diminished secondary aggregation to epinephrine, low molecular ADP, diluted collagen and thrombin, suggesting an impaired release of endogenous ADP (92,93). The defective aggregation to epinephrine and collagen is aggravated by maternal aspirin ingestion (93). The mechanism is unknown, although studies have suggested a defect in release and stores of metabolic ADP (94) and in release of serotonin (95). Electronmicroscopic studies indicate that neonatal platelets are normal and thus have dense bodies, those organelles which contain non metabolic ADP (96). The studies of Stuart (97) and Del Principe (98) have shown that the neonatal prostaglandin synthetic pathway is probably intact (possibly slightly impaired) on stimulation with thrombin. Corby (99) showed that

arachidonic acid induced aggregation of neonatal platelets is normal. It was concluded that the cyclooxygenase pathway is normal. By several weeks of age normal platelet aggregation is found (100). It has been postulated that hypoxia plays an aetiological role in the transient platelet function defect of newborns (88).
Platelet adhesiveness to glass is normal and clot retraction decreased (92, 101). The response of neonatal platelet to ristocetin is normal (96). Several groups of investigators (97,102) have demonstrated that the bleeding time is not prolonged in newborn infants. This test is considered to be the most important clinical test of platelet function (103).

3. Coagulation proteins

a. Contact factors: XII, XI, prekallikrein, high molecular weight (HMW)kininogen.
The contact factors are reduced in normal newborns, with their range being between 20-30% in preterm infants and 20-50% in term infants (104,105,106,107). Except for factor XI deficiency, a deficiency in the contact factors has not been associated with significant haemorrhage in adults. The deficiency of the contact factors in newborns may at least in part explain the extremely long partial thromboplastin time (PTT) frequently seen in normal term and preterm infants (108). In general, a deficiency of the contact clotting factors results in a much longer PTT than a corresponding deficiency of factor VIII or factor IX (91). Different reagents used for the PTT cause normal ranges to vary greatly from one laboratory to another. The sensitivity and normal ranges must therefore be specifically defined in the individual laboratory. According to Montgomery (91) the expected normal range for normal term infants in their laboratory is 55-75 seconds (range in adults 36-51 sec). In our laboratory the normal range for normal term infants is 38-70 sec. (adults: 28-40).

b. Factor V and Factor VIII
Factor V activity levels in all newborn infants are comparable with adults (109,104). Even in extremely premature infants (28 weeks) a mean level of 65% (43-80) has been described (Barnard 104). Also the factor VIII levels appear to be in the normal adult range in most studies. The data are summarized in table 2 for normal term infants.

TABLE II

factor VIII procoagulant activity	125-168%	(110,104)
factor VIII coagulant antigen	25- 65%	(111)
factor VIII related antigen	22-200%	(104,112,113,114)
factor VIII ristocetin cofactor activity	55-140%	(112,114)

It is important to realize that most of the studies have been performed with cord blood specimens. Delivery itself is associated with profound physiological alterations, as may be illustrated by the study of Johnson (115) who found 30-50% higher values of factor VIII-von Willebrand factor complex in vaginally delivered infants, compared to infants delivered by Caesarean section.

Fukui (114) suggested qualitative abnormalities of newborns factor VIII based on crossed immunoelectrophoretic studies. This alteration could not be confirmed by other authors (115).

c. The vitamin K-dependent coagulation factors: II, VII, IX, X.

The vitamin K-dependent procoagulants (F II, F VII, F IX and F X) are reduced in both term and preterm infant. (116,117,109,104). Most studies have been performed using clotting activity assays. These studies show a gestational age dependency. The concentration increases from about 30% at 24 weeks to about 50% of adult normal values at term. Immunological studies of prothrombin (F II) indicate, that no differences exist between newborn and adult prothrombin. In some cases, however, changes associated with a vitamin K deficiency have been described (118). These observations have been refuted by other groups (119,120). The "vitamin K problem" in newborns is extensively discussed in chapter 2 of this thesis.

d. Fibrinogen (Factor I) and fibrin-stabilizing factor (Factor XIII)

Quantitative studies of fibrinogen in term infants indicate that the levels are in the normal adult range (108). The mean values range from 1.9-2.6 gr/l. Aguercif (121) found slightly lower values using clot density or thrombin clotting methods than using colorimetric, heat precipitation or biuretmethods. A number of investigators have suggested the possibility of a distinct fetal fibrinogen, which might explain the prolonged thrombin and reptilase clotting times found in all newborns, in the absence of fibrin(ogen) degradation products. In chapter 7 of this thesis we have reevaluated this so called fetal fibrinogen. We have concluded that most of the differences reported in the literature to be due to a distinct fetal fibrinogen, cannot be confirmed, provided that optimal care is taken to minimize proteolytic breakdown in vitro. The only remarkable difference is the observation that the phosphate content is 3-4 fold higher in cord fibrinogen than in adult fibirnogen. Most of the phosphorus in fibrinogen is located in the part of the molecule where the fibrinopeptides are splitt off. Until now the function of phosphorus in fibrinogen is unclear. In one study (122) dephosphorylation of "fetal fibrinogen" appeared to have no influence on its clotting properties. Some authors have suggested that in cord blood a mixture of adult and "fetal fibrinogen" is present, and that in premature infants relatively more "fetal fibrinogen" is present (123). The different clotting properties of "fetal fibrinogen" have also been ascribed to a higher sialic acid content and it has been postulated that

enzymatic removal of sialic acid corrected the abnormal findings (127). In our study we found the same carbohydrate content in as well adult as cord fibrinogen. Factor XIII levels in normal term infants are approximately 50% of the values found in normal adults (124).

4. Coagulation inhibitors

a. Serine protease inhibitors: Antithrombin III and α_2-macroglobulin

Antithrombin III levels in newborn infants are also reduced compared with adult values, irrespective of the method used (108). Levels range from 40-87% in normal term infants by immunological measurements, from 45-60% by clotting activity methods and from 11-104 (mean 65%) by chromogenic substrate method. The antithrombin III levels are also dependent on the gestational age (126,109,127). McDonald (1982) found in a biochemical and functional study of antithrombin III no differences between adult and newborn antithrombin III (128). In a recent report (129) a reduced activity/antigen ratio of antithrombin III in newborns (range 0.26-0.86) was described. This discrepancy was present in 90% of all samples and independent of the state of health. It could not be attributed to laboratory artefacts, circulating antithrombin III - thrombin complexes or by increased heparin cofactor II plasma levels in neonates. The possibility of an abnormal newborn antithrombin III molecule with decreased heparin affinity was postulated, but not substantiated. Although 50% reductions in antithrombin III have been associated with major thrombotic tendencies in adults, it is of interest, to note that newborn antithrombin III levels are in the same range as the vitamin K dependent coagulation factors. This might explain in part why in normal healthy term newborns thrombosis is rare. The levels of α_2-macroglobulin are normal in normal term infants compared with adult values (130,131) or even increased to around 150% (119). α_2-macroglobulin complexes with serine proteases and relies upon reticuloendothelial clearance rather than inactivation (132). Its physiological role may therefore be reduced because of the newborns reduced reticulo endothelial function (91). In adults antithrombin III contributes to 60-70%, α_2-macroglobulin 20-30% of the total antithrombin capacity. This has not been investigated for newborns.

b. Protein C, Protein S and APC inhibitor

In normal term infants protein C levels are 18-46% of adult values (Marlar 1984, unpublished data, cited in Montgomery (91)(133,134). Protein S levels were 7-40% (91) and APC inhibitor 65-96% (91). Since the role of both protein C and S is to inactivate factors V and VIII, a deficiency of protein C results in a thrombotic tendency (135). Factor V and VIII are both at normal (adult) values in newborns.

The physiological imbalance between protein C and factors V and VIII is therefore a theoretical cause for a thrombotic tendency. Recurrent thrombotic problems or purpura fulminans in the neonatal period, how-

ever, have only been found in cases of a homozygous protein C deficiency (136,137,138). On the other hand, these problems should prompt the clinician to consider this diagnostic possibility because without appropriate therapy homozygous protein C deficiency is probably incompatible with life. It is of interest, to note that for unknown reasons these infants do not die in utero (91).

5. The fibrinolytic system

Most studies suggest an overall increase in fibrinolytic activity in newborns (109), in spite of the fact that plasminogen is reduced to 50% of normal adult values and that α_2-antiplasmin antigen is in the normal adult range (105). The increased fibrinolytic activity may theoretically be due to increased plasminogen activator levels and/or reduced levels of plasminogen activator inhibitors.

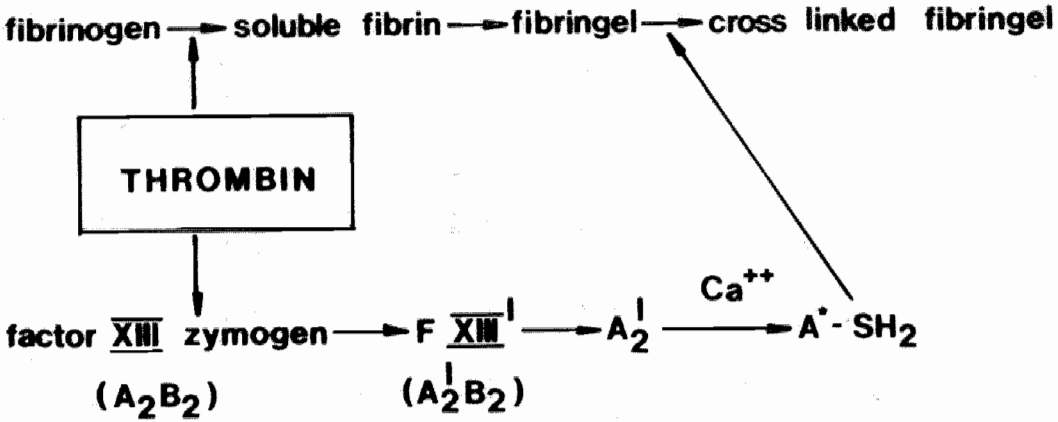
Recently, no studies have been published on this subject in newborns. Beller found in 1966 increased activator activity in cord blood (139), Ekelund (139) found normal values. Our knowledge of the fibrinolytic system in newborns is therefore limited. However, it is important to note that infants stressed with respiratory distress syndrome (RDS) or disseminated intravascular coagulation (DIC) quickly deplete their fibrinolytic potential (140), thereby loosing one of the physiological defense mechanisms against the development of (micro)thrombosis.

6. The prolongation of the thrombin clotting time in newborns

The role of thrombin in the formation of a stabilized fibrin gel is summarized in figure 2. Thrombin cleaves the negatively charged fibrinopeptides A and B (FPA and FPB) from the amino-terminal positions of the α and β chains. The resulting change in charge distribution over the molecule, now called fibrin monomer and the exposure of two sets of polymerization sites permit the polymerization of fibrin into fibers and networks (141). A final modification to ensure the stability of the fibrinclot is provided by the activation by thrombin of factor XIII. This factor catalyzes the formation of irreversible covalent bonds between glutamic acid and lysine residues on adjacent fibrin monomers (142), the so called cross linking. This is shown in figure 2.

A prolonged thrombin clotting time in plasma is found in quantitative and qualitative abnormalities of fibrinogen and when inhibitors are present of thrombin, of fibrin monomer polymerization and of fibrin gel cross linking. Thrombin clotting times are measured by adding 50 µl of a thrombin solution of 5-10 N.I.H.U/ml to 0.2 ml platelet free plasma after 30" incubation of the plasma at 37 °C. It is a well known observation that the thrombin clotting time in all normal term newborns is prolonged. This observation could not be attributed to quantitative and or qualitative alterations of fibrinogen (chapter 7 of this thesis) or

FIGURE 2

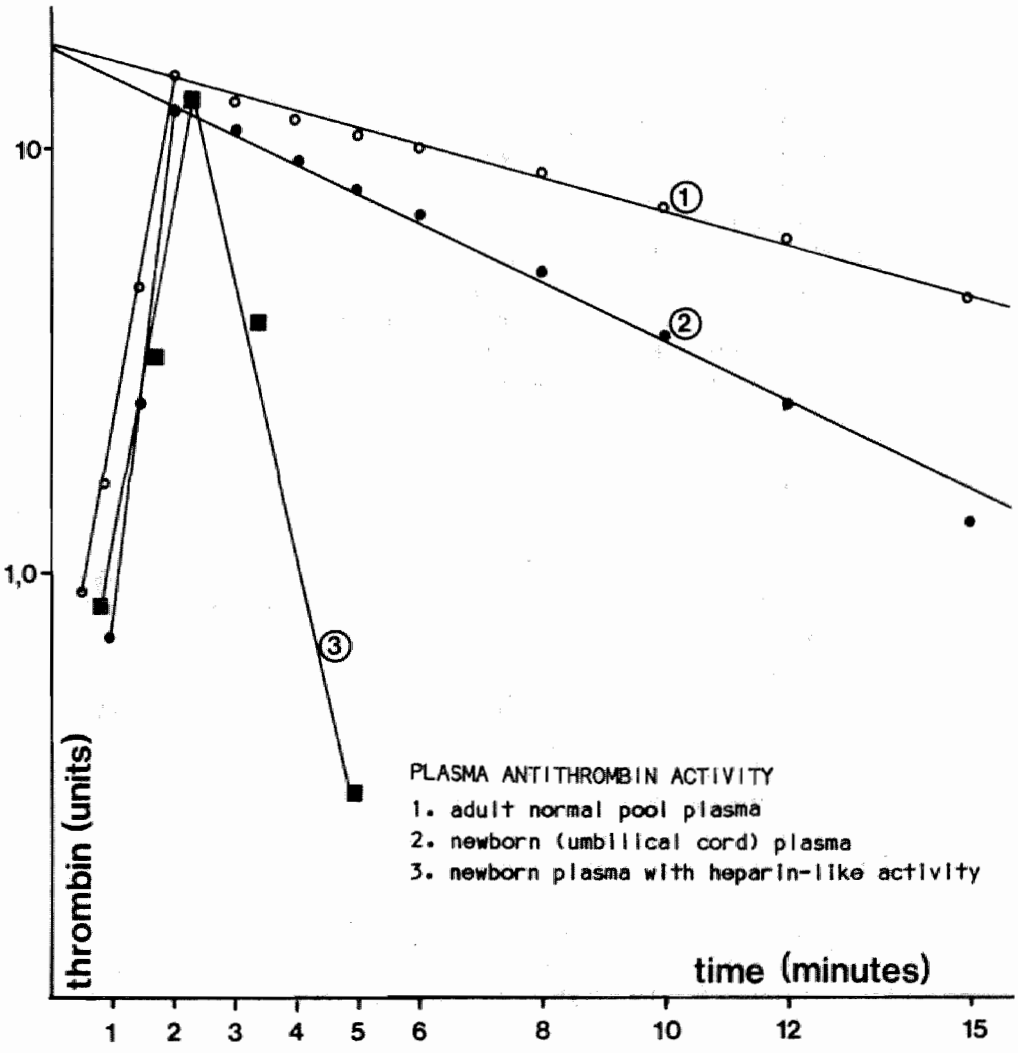


The formation of a stabilized cross-linked fibringel

large amounts of fibrin(ogen) degradation products.
Using the conventional assay (Wellcome) we found in none of the samples more than 10 mg/l FDP's, however, it can not be excluded that smaller amounts contribute to the prolongation of the thrombin clotting time in newborns. In chapter 3 of this thesis we describe the presence of small amounts of fibrin- and fibrinogen degradation products in a number of cord plasmas, in which inhibitory material was present as judged from thrombotest dilution curves.
In a very few cases, a remarkable prolongation of the thrombin clotting time was found. In 2 out of 200 samples the thrombin clotting time was longer than 5 minutes (normal range cord plasma: 23-46"). These samples were obtained from infants, who differed in no way from the other investigated newborns. In these samples we found an alternative explanation for the prolonged thrombin clotting time, suggesting a heparin-like substance. In figure 3 the curves are shown of a two stage prothrombin assay of adult and cord plasma. In these curves the formation of thrombin in plasma and its disappearance due to antithrombin activity present in plasma is shown. They were performed as described by Hemker et al (143). From this figure it can be seen that there are only slight differences between adult and newborn plasma. However, in the 2 newborn samples with a remarkable prolonged thrombin clotting time, the thrombin generation curve was markedly different so that thrombin was not any longer demonstrable already after 5 minutes. These 2 newnborn samples were further investigated. The thrombin inhibitory effect could be reduced by adding calcium and protamine chloride to the plasma. Because of the limited quantity of these plasma it has not been possible to purify and characterize this inhibitor. Therefore, we have been unable to check the data obtained as thoroughly as we would like to. All fibrinogen related material was removed using a ristocetin-sepharose column. In the eluate the inhibitor was present and could be adsorbed to a protamine-sepharose column. The inhibitor was eluted from this column using a sodiumchloride gradient. This eluate was extensively dialyzed against buffer (0.15 M NaCl, 10 mM Tris-HCl, pH 7.4). The inhibitory effect was measured by incubating of the inhibitory sample with normal adult plasma and measuring a thrombin clotting time. The inhibition was abolished by adding protamine chloride and upon incubation with heparinase, (from cultures of the flavobacterium heparinum), which was kindly provided by Dr. Rosenberg. The inhibition could not be abolished upon incubation with trypsin. The inhibitory effect appeared to be dependent on the presence of antithrombin III. In the following experiments antithrombin III (Kabi Vitrum, Amsterdam, the Netherlands) (f.c. 1 U/ml) was added. The effect of the inhibitor on the amidolytic activity of thrombin and factor Xa was measured in a Beckman spectrofotometer, using the chromogenic substrates S2238 and S2337. As a reference, commercial heparin (thromboliquine, Roche, Basle, Switzerland) was used (1

FIGURE 3

TWO STAGE PROTHROMBIN ASSAY



I.U./ml). The inhibitor from newborn plasma had no effect on the amidolytic acitivity of factor Xa (f.c. 20 nM), however, a remarkable effect of the activity of thrombin (f.c. 0.1 N.I.H.U./ml). The reference commercial heparin showed a complete inhibition of factor Xa in concentrations which gave less inhibition of thrombin than the newborn inhibitor. The above mentioned experiments suggest that in a few cases (1%?) of normal term infants heparin-like activity can be demonstrated with a remarkable anti IIa and hardly any anti Xa effect. Until now, heparin-like activity has only be demonstrated in a few case reports of patients with haematological malignancies (144). To our knowledge, such undeniable heparin-like activity in normal individuals has never been demonstrated.

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CHAPTER 2

VITAMIN K AND THE NEWBORN

1. Introduction
2. Vitamin K deficiency in newborns: possible mechanisms
3. How to diagnose a vitamin K deficiency
4. The situation before, at and after birth
5. Conclusions and recommendations

1. Introduction

Studying the effects of a cholesterol-free diet on chickens, Dam (1) noticed that after some weeks the animals developed a haemorrhagic diathesis, which could not be counteracted by adding purified cholesterol to the food, but turned out to be related to the deficiency of a fat soluble vitamin, which was called vitamin K (Koagulations Vitamin). It was postulated that vitamin K was essential for the synthesis of a number of proteins involved in blood coagulation. Vitamin K was also involved in the haemorrhagic disease in cattle, known since 1921 in the USA and Canada. Campbell and Link (2) have demonstrated that this disease was caused by the presence of coumarin derivatives in sweet clover hay. These 4-hydroxy coumarins are antagonists of vitamin K. It would take more than 30 years after these initial observations to gain more insight in the mode of action of vitamin K and to determine at what stage of the protein synthesis vitamin K is involved. The first breakthrough came in 1963, when Hemker et al. described that blood from patients receiving oral anticoagulant therapy contains a mixture of normal and abnormal prothrombin (3a). This abnormal prothrombin was first called preprothrombin (3a) and 5 years later "Protein Induced by Vitamin K Absence" or PIVKA (3b) and it was regarded as a precursor of prothrombin. It was postulated that this abnormal prothrombin could not be activated to form thrombin under physiological conditions. It was shown that abnormal prothrombin is similar to normal prothrombin with respect to its molecular size, its number of sialic acid residues, its amino acid composition and its antigenic activity (4). However, in the presence of Ca^{++}, the electrophoretic migration rate of abnormal prothrombin was different. This appeared to be due to the fact that abnormal prothrombin is not able to bind Ca^{++} whereas prothrombin has a high affinity for Ca^{++} (5). In 1974 it was reported independently by two groups (6,7), that the calcium-binding sites in normal prothrombin were gammacarboxyglutamic acid (Gla) residues, and that these residues were glutamic acid (Glu) residues in abnormal prothrombin. The only known physiological significance of Gla residues is related to their Ca^{++} binding properties. For instance, the formation of thrombin is enhanced in the presence of Ca^{++} and negatively charged phospholipids (8). Vitamin K dependent proteins are also involved in the calcium transport from the egg shell to developing chicken embryos (9) and in the regulation of the calcium phosphate deposition in the bone matrix (10). All vitamin K-dependent proteins discovered up till now belong to what are called the secretory proteins: the proteins which are secreted after maturation into the extracellular fluid. Vitamin K is involved in a carboxylation reaction, which is a posttranslational step in the synthesis of Gla-containing proteins (11). Enzymes involved in posttranslational modifications are located in the inner side of the endoplasmic tubular system. The vitamin K-dependent carboxylase has been studied most extensively in liver microsomal preparations obtained from rats and cows. Absolute

requirements for the vitamin K-dependent carboxylase are vitamin K hydroquinone (or vitamin K quinone in the presence of reducing agents such as NAD(P)H), O_2, CO_2 and a carboxylatable substrate. Several stimulators and inhibitors have been described, mainly based on in vitro studies.
In the liver, vitamin K is partly present in the form of its 2,3 epoxide (12). The epoxide can be reduced to vitamin K quinone by vitamin K epoxide-reductase and further to the hydroquinone by NAD(P)H dehydrogenase (13) and/or vitamin K reductase. This course of events is called the vitamin K cycle. The biological function of the vitamin K cycle is thought to be the constant preservation of vitamin K hydroquinone, which is used in the carboxylation reaction. Carboxylation without epoxidation has never been demonstrated and the concept that both events are coupled in some way has generally been accepted at this time (11).
During the last few years, a number of investigators has reported the presence of vitamin K-dependent carboxylase in non-hepatic tissues (10,17, 18,19). Microsomal carboxylases and substrates were found in bone, testis, kidney, spleen, lung, thyroid, pancreas, thymus, placenta and arteries; it has not been found in veins, brain, leukocytes and muscles. The importance of these carboxylases as well as the function of the carboxylated reaction products remains to be established as yet. The vitamin K-dependent proteins involved in the haemostatic reaction are all synthesized in the liver. The four vitamin K-dependent coagulation factors are involved in the formation of thrombin from prothrombin. Thrombin acts as a key enzyme in the haemostatic mechanism. It converts fibrinogen into fibrin and it activates factor XIII, which in its activated form provides a crosslinking of fibrin monomers. More important is the fact that thrombin in vivo as well as in vitro is a potent activator of blood platelets. It also stimulates its own formation by activation of two other coagulation proteins, the factors V and VIII. Protein C is a vitamin K-dependent zymogen, which, when activated, functions as an anticoagulant. The physiologic activator of protein C is believed to be thrombin in complex with an endothelial cofactor, thrombomodulin (20). Activated protein C proteolytically degrades coagulation factors Va and VIIIa (21) and also elicits fibrinolytic activity in plasma (22). Protein S is another vitamin K-dependent anticoagulation factor which acts by increasing the rate of inactivation of factor Va by activated protein C. Deficiency of protein C as well as protein S, is associated with an increased risk for thrombosis (23,24).

2. Vitamin K deficiency in newborns: possible mechanisms.

Vitamin K deficiency may occur in both acutely ill and healthy appearing neonates and can be encountered in a wide variety of clinical situations. This is summarized in table I.

TABLE I

Conditions associated with vitamin K deficiency in infants and neonates

	Ref. no.
1 maternal drug ingestion	25,26
2 decreased exogenous intake (dietary intake)	27,28,29,30
3 decreased endogenous production	
- antibiotic therapy	31,32
- ? slow gut colonization	
- ? gut colonization by non vitamin K-producing bacteria	
4 Malabsorption of fat	
- infectious diarrhoea	27,30,32,33
- intestinal resection	34 (p 924)
- cholestyramine administration	34 (p 1103)
- cystic fibrosis	35
- cholestatic liver disease (e.g. biliary atresia, α_1-antitrypsin deficiency)	36
- abetalipoproteinaemia	37

According to some authors another possibility is failure to administer vitamin K_1 at birth (38). However, it is not to be expected that one dosage of vitamin K at birth is able to provide protection for more than a few days, because the half life time of the vitamin is short (2-4 hours). Even, if one assumes, that vitamin K is recycled many times in the tissues and that dosages are given far above the physiological requirements, it is hard to believe that the effect will last for more than one week. Another important feature is the placental transfer to vitamin K.

Recently it has been demonstrated by several authors (39,40) that there is a placental barrier of the transfer of vitamin K_1. From these studies, it may be concluded, that when the vitamin K supplies are low in the mother the fetus is relatively more at risk for developing a vitamin K deficiency and that repeated and relatively high dosages are required to correct this state. This could be a possible explanation for the fact that a vitamin K deficiency is more frequently found in for instance South East Asia, Japan and South Africa (blacks) and is probably due to a lower socioeconomic status in general (41,42). In these countries a vitamin K deficiency is found at birth as well as in the first 6 months of life. However, also in the western world a number of reports have been published, which indicate an increased incidence of the haemorrhagic disease of the newborn, a disease first described by Townsend in 1894 long before the discovery of vitamin K (43).

The classical haemorrhagic disease of the newborn occurs in the first week of life, independent of trauma, infection or anoxia after an uneventful pregnancy and delivery in apparantly normal healthy newborns. In the recent literature, the increased incidence of this disease has been attributed to the increase in alternative birthing and breast feeding (38,44,45), which might favor the development of a vitamin K deficiency. Another haemorrhagic syndrome occurs in newborns between 1 and 6 months after birth, which is almost exclusively found in breast-fed infants and also attributed to a vitamin K deficiency (28,32,41,46). There is also a tendency in the recent literature to recommend prophylaxis with vitamin K to all newborns at birth as well as in the first months of life in breast-fed infants, in spite of the fact that especially in the western world only a small minority of the patients will develop a vitamin K deficiency. In a recent survey from Japan the incidence of vitamin K deficiency was 1 : 4500 unselected infants and 1 : 1700 breast-fed infants (47). However, intracranial haemorrhage was present in 81% of the affected children. This is seen as a strong argument in favor of the vitamin K prophylaxis in all newborns and especially in breast-fed newborns. In previous work of our laboratory, no evidence of a vitamin K deficiency in healthy full term infants at birth and in the third day of life could be demonstrated (57). It was concluded that vitamin K should not be given to all newborns, but on indication in high risk groups. Recent literature data caused us to reassess our opinion on the vitamin K problem in newborns. Therefore, we tried to answer the following questions:

- How to diagnose a vitamin K deficiency?
- Which factors determine the vitamin K deficiency, described in newborns at several time points before and after birth?
- Does vitamin K prophylaxis reduce the incidence of major bleeding problems in newborns and should all babies therefore receive vitamin K prophylaxis?

3. How to diagnose a vitamin K deficiency

Coagulation abnormalities are commonly observed in ill newborns. Haemorrhagic problems in acutely ill newborns occur most commonly with thrombocytopenia due to sepsis and disseminated intravascular coagulation (DIC) (44). In contrast, an apparantly healthy newborn with haemorrhage should be suspected to have a vitamin K deficiency (48). The laboratory diagnosis of a vitamin K deficiency depends not only on the methods used in the laboratory but also on the quality of the sample. It will be clear that obtaining adequate material from newborns is a difficult task. In our experience newborn blood is more suspectible for in vitro (and/or in vivo?) proteolytic breakdown, in particular that of fibrinogen. Therefore we recommend the blood to be collected in Hepes buffered citrate to which aprotonine is added and the plasma rendered platelet free by a second run at 20.000 g. Another important factor is the haematocrit. In the first week of life the haematocrit values can show substantial differences and it is

reported that in 10% of all newborns haematocrit values of 0.60 and even higher are found (49). In polycythaemia false prolongation of the prothrombin time has been described due to an excess of citrate. Many authors use the prothrombin time or one of its modifications, the Normotest® and Thrombotest®, as evidence for an underlying vitamin K deficiency. Although a prolongation of the clotting times obtained with these assays can be caused by a vitamin K deficiency, also other factors can be responsible, such as proteolytic degradation products and high haematocrit values. We have found a poor correlation between prothrombin coagulation activity levels, Thrombotest® values and Normotest® values, thus indicating the limited validity of these overall clotting tests in the diagnosis of a vitamin K deficiency, unless the values are extremely low. These tests are therefore less reliable for providing evidence for a vitamin K deficiency in newborns or that vitamin K given to newborns "improves" the coagulation abnormalities, as is often done, especially in the older literature. Prothrombin levels can be measured more reliable in several ways. In the one stage assay using human prothrombin deficient plasma (50) the biologically active prothrombin is measured.
In the following tests total prothrombin protein is measured, including that which is functionally inactive (descarboxy-prothrombin) which is expected to be present in plasma in the case of a vitamin K deficiency:

1. The one dimensional rocket immunoelectrophoresis according to Laurell measures the factor II related antigen using a precipitating antibody (51).
2. The Echis Carinatus test, using saw-scaled viper venom, which enzymatically activates prothrombin into thrombin in the absence of lipid and calcium (52).
3. The staphylocoagulase reacting factor assay (53).

In most studies a discrepancy between the factor II clotting activity level and the factor II antigenic level via method 1 is taken as evidence for a vitamin K deficiency, when the factor II_c/factor II_{ag} is below 0.8. This method is rather insensitive and requires at least 5% descarboxy-prothrombin present in the sample. Another immunological method often used is the two dimensional crossed immunoelectrophoresis. In the first dimension calcium lactate is added (5 mM/l) in the running buffer. Prothrombin and descarboxy-prothrombin have a different migration pattern, which can be visualized in the second dimension where a human precipitating antibody against prothrombin is used. Because descarboxy-prothrombin does not bind Ca^{++} it migrates faster to be anodal site (54). This method is not quantitative and also rather insensitive. As yet the most sensitive method for the determination of descarboxy-prothrombin has been described by Furie, using a radioimmunoassay with a specific antibody against descarboxy-prothrombin (55). Recently this method was used to evaluate the vitamin K status of newborns and their mothers (63). They found a mean

level of descarboxy-prothrombin of 0.35 µg/ml (0.001-78 µg/ml) in newborns and 0.06 µg/ml (0.001-1.01 µg/ml) for the mothers, whereas it was undetectable in normal subjects. The results were obtained in paired cord serum samples of 181 term newborns and their mothers at delivery. In one Japanese report (56) an enzyme-linked immuno-sorbent assay using monoclonal antibodies against descarboxy-prothrombin was applied in 4 infants, ranging in age from 27-42 days with suspected vitamin K deficiency. In these infants the levels of descarboxy-prothrombin were much higher than in the controls. Another approach was published by Atkinson (42) who devised a rapid and sensitive spectrophotometric assay based on the ability of disphollidus typus venom to activate prothrombin remaining in plasma absorbed with aluminium hydroxide gel. The non absorbable descarboxy-protein was expressed as rate of reaction of non absorbed plasma x 100. The authors claim that descarboxy-prothrombin levels can be detected via this method up to 0.1% of the total prothrombin level. They found detectable descarboxy-prothrombin in 48 out of 128 umbilical cord plasma samples.

Many authors emphasize the importance of the effect of the administration of vitamin K as a diagnostic tool for a vitamin K deficiency state. Recently, it has become possible to measure directly the vitamin K_1 levels in plasma, using HPLC method after lipid extraction (39). In this study, the majority of the umbilical cord plasma samples appeared to have low or even non-detectable amounts of vitamin K_1 (39). This has been taken as a definite evidence for the occurrence of a vitamin K deficiency in the majority of all newborns. However, determination of vitamin K_1 in plasma is still a controversial subject. It may also be questioned if this method reflects the amount of vitamin K in the target tissues and if for instance in newborns the low levels can also be explained by the markedly lower levels of the transport lipoproteins and albumin or a markedly higher extraction of vitamin K_1 from newborn plasma in the peripheral tissues. Moreover, the amount of vitamin K required is unknown and vitamin K_1 represents not the total vitamin K pool. Up till now these interesting results have not been confirmed by other groups.

In our laboratory we use as a screening procedure for a vitamin K deficiency the ratio of IIc (prothrombin clotting activity) levels and total prothrombin levels as measured immunologically (rocketimmunoelectrophoresis) or by the Echis Carinatus test.

4. Factors which may determine vitamin K deficiency

In humans, the daily requirement of vitamin K is estimated to be very low. For adults it is supposed to be in the range of 0.05-1.5 g per kg weight per day (58). Food and the intestinal bacterial flora may serve as sources for vitamin K. Vitamin K_1 (phylloquinone) is thought to be the most important form and is synthesized by green plants. Therefore, it is mainly present in vegetables. The intestinal bacterial flora produces the family of the K_2 vitamins, the menaquinones, which differ from each other by their

side chain length. Up till now, only vitamin K_1 epoxide has been demonstrated in human plasma (59). However, K_2-vitamins have been demonstrated in the liver and are said to form 50% of the total vitamin K pool (60). Furthermore, in adults deprived of food, a vitamin K deficiency is rarely found, unless also the bacterial flora is disturbed for instance by using antibiotics. The role of the K_2 vitamins in the synthesis of the vitamin K-dependent (anti)-coagulation factors remains unclear. In newborns the daily requirement of vitamin K is unknown, but it might be higher than in adults. It could be that newborns utilize much more vitamin K for the synthesis of non-hepatic vitamin K-dependent proteins, such as the bone Gla-protein osteocalcin, which is involved in the regulation of calcium precipitation in the bone matrix and the synthesis of which is remarkably high in rapidly growing bones (62).

4a. The situation before birth

Only a few studies have been performed to assess the amount of the vitamin K-dependent coagulation factors before birth. These data are summarized in table 2.

TABLE II

	gestational age (weeks)	Coagulation factors (units/ml) IIc	IIag	VII	X	IX	sampling*
Heinkinheino(64)	12-16	0.38	-	0.18	-	-	1
Fortune(65)	11-22	-	-	-	-	< 0.01	1
Holmberg(66)	12-24	-	-	-	-	0.14-0.40	1
Mibashan(67)	16-22	-	-	-	-	0.08-0.18	2
Terwiel(68)	18-24	0.16	0.19	0.21	0.19	0.04	1

*
1. Material obtained via abortion
2. Material obtained via fetoscopy

Terwiel found in the majority of the fetal samples no discrepancy between different factor II determinations. In one sample a small amount of factor II related antigen was observed, compatible with the presence of a small amount of descarboxy-prothrombin or indicative for activation of the sample leading to proteolytic breakdown of prothrombin. Activation of the blood samples appears to be a major problem in the interpretation of the values found, especially in the first reports on fetal development of haemostasis. We were able to study a small number of plasmas obtained via fetal blood sampling in utero. These samples were kindly provided by Dr. F. Forestier, (Hôpital N.D. de Bon Secours, Paris). This group has developed a new and safe method for fetal blood

sampling in utero, using ultra sound guidance of the needle and aspiration of the blood from the umbilical vein at the placental cord insertion (69). In their hands this method has proved to be safe (no abortion in more than 400 cases) and to provide adequate material for haemostatic studies. We could confirm their results and found no evidence for the presence of descarboxy-prothrombin in fetal plasmas. The prothrombin levels gradually increased from 19% (week 21) to 32% (week 33).

Summarizing the scarce data available, no evidence is present at the moment for a vitamin K deficiency during pregnancy in normal healthy infants in the western world, provided that the mother is not vitamin K deficient. In the western world only a small number of pregnant women is at risk for a vitamin K deficiency, for instance by nutritional deficiency, disturbance of intestinal resorption and the use of drugs interfering with the vitamin K mechanism, such as the coumarin derivatives and anticonvulsant drugs.

4b. The situation at birth

It has been well established that all the vitamin K-dependent procoagulants (the factors II,VII,IX and X) are reduced in both the term and preterm infant (70,66,71,72,73,74). In table III the range observed or the mean values ± standard deviations from these studies are given of the vitamin K-dependent coagulation factors in preterm (27-31 weeks) and term infants (38-41 weeks) at birth as a percentage of the normal adult values. These data are based on clotting activity assays.

TABLE III

	preterm infants 27-31 wks	term infants 38-41 wks
factor II (prothrombin)	30 ± 8	54 ± 15
factor VII	24 - 76	35 - 82
factor IX	17 - 20	21 - 39
factor X	14 - 70	33 ± 13

Most studies on the vitamin K-dependent coagulation factors in newborns have been focused on the prothrombin (factor II) levels. In all studies a quantitative reduction of the prothrombin levels is found, using clotting activity as well as immunological methods. In the case of a vitamin K deficiency uncarboxylated precursor molecules are secreted into the circulation, for instance descarboxyprothrombin. The methods available at the moment to assay the presence of descarboxyprothrombin have been discussed in the section "how to diagnose a vitamin K

deficiency" of this chapter. Whether at birth descarboxyprothrombin can be demonstrated is controversial in the literature.
In most West-European studies (74,75,76,77) descarboxyprothrombin could not be demonstrated. This has been refuted by Muntean (78) who found a slightly altered prothrombin peak on crossed immunoelectrophoresis, compatible with changes usually seen in vitamin K deficiency in a small number of "normal" infants. Recently other reports have been published in the United States (80,63,42) and South Africa (42) indicative for the presence of low amounts of descarboxyprothrombin in umbilical cord plasma, using newer and more sensitive methods. These methods detect descarboxyprothrombin in concentrations as low as 0.01-1% of the total prothrombin. In the study of Shapiro et al. (79) umbilical cord plasmas of 917 newborns were investigated. This method is based on the presence of prothrombin related antigen after two adsorption steps with bariumcitrate. Descarboxyprothrombin is not bound to bariumcitrate in contrast to prothrombin. The authors claimed that all normal prothrombin can be removed by this procedure. In 2.9% of the cases descarboxyprothrombin (i.e. prothrombin related antigen after bariumcitrate) could be demonstrated. The incidence was slightly higher in infants born of mothers with hypertension and diabetes mellitus and those who had received antibiotics. The incidence was also higher in infants with infectious complications at birth, respiratory distress syndrome and polycythaemia. In normal infants born of normal mothers the incidence was less than 1%. Atkinson found in his study (42) descarboxyprothrombin in 36% of the cases, using a more sensitive method (detection limit 0.1% of the total prothrombin level). In the study of Blanchard (63) 90% of all newborns (N=181) had detectable descarboxyprothrombin in cord serum, using the most sensitive method available at the moment (RIA with a specific antibody against descarboxyprothrombin). In the South African study (42) especially black infants appeared to have detectable descarboxyprothrombin in their umbilical cord plasma. The authors hypothesize that this might be due to different food patterns and socioeconomic status of the black population. In the other studies these factors are not considered. Although it may be questioned, whether these very low amounts of descarboxyprothrombin are important at birth, it could be that these infants are more at risk to develop a clinical relevant vitamin K deficiency after birth.
Infants born of mothers with convulsive disorders, who have been receiving anticonvulsant therapy are likely to develop a vitamin K deficiency (80). To prevent this complication oral vitamin K should be administered to the mother for the last two months of pregnancy (81). There is no doubt that these infants should be given vitamin K as soon as possible after delivery. In the study of Shapiro (79) an incidence as high as 25% of detectable descarboxyprothrombin has been found in these group of infants.

Infants born to women taking rifampin and isoniazid during pregnancy may also be at risk for early haemorrhagic disease of the newborn (82).

4c. The situation after birth

The situation after birth is more complicated. Most studies are based on clotting activity assays of prothrombin or modifications of the prothrombin time. In none of the studies descarboxyprothrombin has been measured in a sufficient number of infants. Van Doorm (75) found in 5 samples obtained on the third day of life of normal newborns no evidence for the presence of descarboxyprothrombin. Many problems are encountered in studying coagulation abnormalities in newborns in the postnatal period, such as relatively large amounts of blood required and the skill necessary to perform a clean venapuncture. As the newborn blood appears to be more susceptible for in vitro proteolysis, appropriate inhibitors should be present and the amount of the anticoagulant should be corrected for the elevated haematocrit often found in newborns in the first week of life.

In many studies these important features are not mentioned. However, also in studies that fulfil these criteria, a remarkable broad normal range of the values is found. In one study several coagulation and fibrinolytic parameters were measured using micromethods and chromogenic substrates to obtain normal values in the first week of life in a population of dutch newborns (83). In this study a gradual increase of prothrombin levels and an unexplained decline of factor X levels was found. These results, were not influenced by the administration of vitamin K. A gradual increase of prothrombin levels after birth has also been described by other authors (84,70). According to Abalil (85) however, a remarkable decrease of the vitamin K dependent factors is present in most newborns by two to three days of age, rising later in the first week of life secondary to increased oral intake and possible production of vitamin K by the infants' newly-obtained colonic microflora. It was also claimed that this "postnatal depression" can be largely eliminated if the infant receives vitamin K. A number of authors have also found a significant higher prothrombin level in the first days of life of infants that had been given vitamin K. The favourable effect of vitamin K prophylaxis was claimed by a number of authors (85,86, 87,88). However, the administration of vitamin K to newborns did not result in differences with regard to bleeding problems in studies of other authors (89,90);

This has been in part explained by "immaturity" or "defective" hepatic proteins synthesis (91). This hypothesis can be challenged by the well known observation that other coagulation proteins, produced by the liver, which are not vitamin K-dependent, such as factor V and fibrinogen, are within the normal adult range in term infants as well as in premature infants (92).

Recently an increasing number of case reports has been published to emphasize the resurgence of the haemorrhagic disease of the newborn (HDN). In 1952 Dam noted that this disease ocurred more often in breast fed infants (93). This was confirmed by other authors (94,87,95). It was observed that HDN occurred almost exclusively in breast fed infants. Several reasons can be given for the resurgence of HDN in the last five years. First, in recent years there has been a progressive increase in the proportion of babies breast-fed for at least the first two weeks of life (38,96). The vitamin K_1 content of human milk is around one-fourth the amount of vitamin K_1 found in cow's milk (97). Among other possible explanations are: breast milk contains an inhibitor to vitamin K or prothrombin, or the intestinal flora of breast-fed infants is less active in producing vitamin K than the flora of formula-fed infants (95). It is well established that the intestinal flora and stool pH of breast-fed infants differs significantly from that of infants fed with cow's milk. In breast-fed infants 79% of the fecal bacteria are Lactobacillus bifidus and the stool pH is 5.5. The stools of cow's milk-fed infants show a more mixed flora including Lactobacillus bifidus, Escherichia Coli, Enterobacter and Staphylococcus aureus. The breast-fed infant lacks a significant population of vitamin K producing bacteria, such as Escherichia Coli and Staphylococcus aureus (98).

5. Conclusions and recommendations

We conclude that the administration of vitamin K at birth to healthy full term infants is only indicated when the mother is vitamin K deficient (or when this is suspected), for instance resulting from fat absorption disturbances, lenghty use of antibiotics, malnutrition and if the mother has used drugs with a known anti-vitamin K effect such as the coumarin derivatives or anticonvulsives.
It may be considered to administer vitamin K also to infants born of hypertensive or diabetic mothers, to infants born after a complicated delivery and to infants with infectious complications and respiratory distress syndrome. Whether all infants small for gestational age and premature infants should be given vitamin K prophylaxis remains to be established. Other well defined risk groups of infants who should receive vitamin K prophylaxis include: infants who require total parenteral nutrition or prolonged intravenous therapy, infants who have chronic diarrhoea, cystic fibrosis or biliary atresia or other diseases known to be associated with a malabsorption of dietary vitamin K and infants receiving antibiotics or other drugs known to interfere either with the vitamin K-producing intestinal flora, the absorption of vitamin K from the intestine, the transport of vitamin K to the liver or the availability of vitamin K in the hepatocyte. Vitamin K prophylaxis at birth as well as in the postnatal period is strongly propagated,

especially in the literature from the United States for all newborns, especially those exclusively fed with human milk. In The Netherlands a clinical relevant vitamin K deficiency in newborns is very rare. Therefore we do not recommend the routinely use of vitamin K prophylaxis for all newborns. However, vitamin K prophylaxis should be considered especially in breastfed infants, when additional risk factors are present.

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CHAPTER 3

FIBRIN(OGEN) DEGRADATION PRODUCTS IN NEWBORN PLASMAS CAN CAUSE A FALSE INTERPRETATION OF THE PROLONGATION OF THE THROMBOTEST CLOTTING TIME

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Summary

According to the literature, clotting inhibiting material is present in umbilical cord plasmas of 67% of all healthy full term infants as judged from thrombotest dilution curves. We investigated 40 umbilical cord plasma samples, which were drawn under conditions with minimize in vitro activation of the haemostatic mechanism.
In these samples, the prothrombin clotting activity (IIc) levels, using a one stage method, ranged from 28.8 - 73.7% as compared with adult normal plasma values (100%). The mean value was 50.6% ± 12.1. There was no difference between the inhibited (in the thrombotest dilution curve) and non-inhibited group with regard to the prothrombin levels (IIc).
The thrombotest clotting times ranged from 35.2 - 66.5 sec (53.3 ± 8.1). The correlation coefficient (r-value) between the thrombotest clotting times and the prothrombin (IIc) levels was -0.46.
Using sensitive immunoassays for fibrin degradation products (XDP) and fibrinogen degradation products (FDP) based on monoclonal antibodies, we found that no degradation products could be demonstrated in the non-inhibited group, whereas degradation products were found in the inhibited group.
The most striking finding was the presence of fibrinogen degradation products. The prolongation of the thrombotest clotting time could be imitated by adding small amounts of purified fibrinogen degradation product fragment X to umbilical cord plasma with a normal thrombotest clotting time. We conclude that there is a poor correlation between the thrombotest clotting time and prothrombin activity (IIc) levels in newborns. This makes the thrombotest of less value in the assessment of the vitamin K-dependent coagulation factors in umbilical cord plasma. Furthermore, we conclude that this poor correlation can be explained by the presence of small amounts of fibrin(ogen) degradation products, which are undetectable using conventional assays for FDP and/or XDP.
As utmost care was taken to avoid proteolytic breakdown in vitro, our findings most likely reflect an enhanced fibrino(geno)lytic activity in umbilical cord plasma in vivo.
This could be due to an increased tissue-plasminogen activator activity in umbilical cord plasma. The enhanced tissue-type plasminogen activator (tPA) activity may be due to an enhanced release from the endothelial cells and/or a decreased inhibition by a recently discovered fast-acting inhibitor of tPA.

Introduction

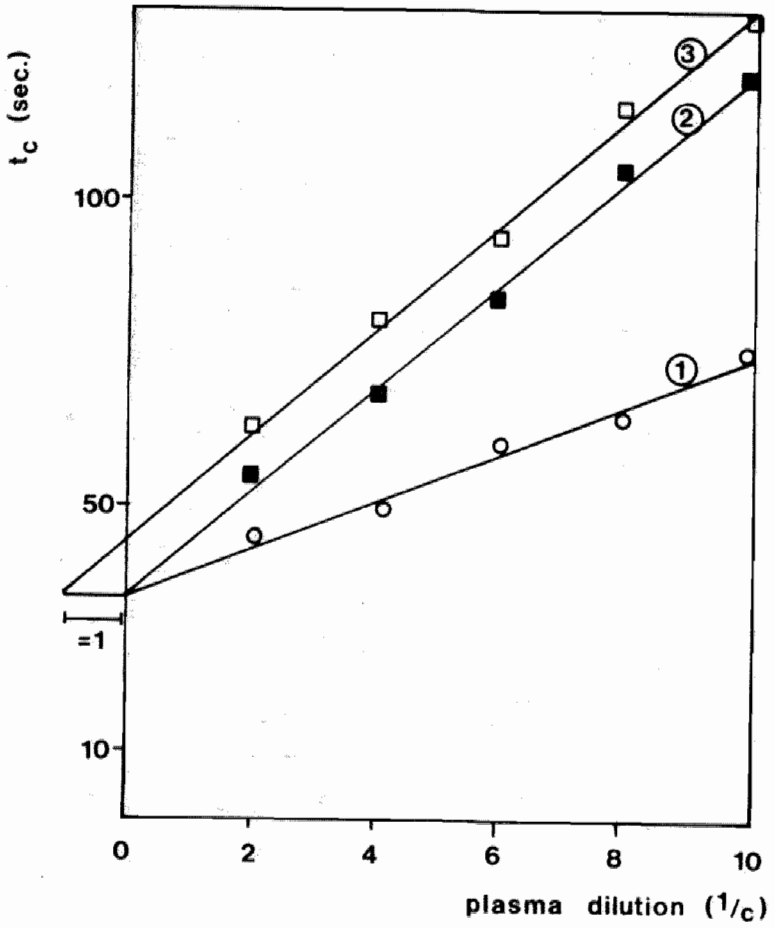
The vitamin K-dependent coagulation factors in full term, healthy newborns are at birth in the range of 20-60% of the values found in adults (1). A vitamin K deficiency can be held responsible for this in only a minority of

the cases (2,9). Among the laboratory tests, often used in clinical practice, to obtain an impression of a deficiency of the coagulation factors II, VII and X are Thrombotest® and Normotest®. Both reagents are commercially available (Nyegaard, Oslo, Norway) and contain $BaSO_4$-adsorbed bovine plasma and a source of thromboplastin. The exact composition of these commercial reagents has not been published.

The tests are variants of the classical prothrombin time test in the presence of excess factor V and fibrinogen. Therefore, a possible deficiency of factor V and/or fibrinogen is not measured in the thrombotest or normotest assay. The tests are also insensitive to a deficiency of factor IX, because of the large amount of thromboplastin, present. Moreover, a number of other factors, besides the amount of the coagulation factors II, VII and X, influence the clotting times. Relatively short thrombotest clotting times are found, when the blood samples are stored for more than 2-4 hours in glass tubes at low temperatures. This phenomenon is probably due to a factor XII dependent activation of factor VII (3) and is especially important in plasmas of women, using oral contraceptive drugs (4).

Relatively long thrombotest clotting times are found, whenever one of the coagulation factors II, VII and X becomes rate limiting, for instance when one of these factors is congenitally deficient or early in oral anticoagulant therapy with coumarin derivatives. Other causes for relatively long thrombotest clotting times are for instance the presence of a structurally abnormal factor IX (Haemophilia B_M) (5), the presence in plasma of heparin, descarboxy-coagulation factor X (6) and an excess of citrate (7). Descarboxy-coagulation factors II, VII and X are found in plasma of vitamin K deficient patients and/or in the plasma of patients using coumarin derivatives as oral anticoagulant therapy.

The inhibitory effect of the descarboxy-factors on the thrombotest clotting time was described by Hemker et al for the first time (1963) and provided the basis for the modern concept of the mechanism of action of vitamin K (8). It is not known, if this inhibition has any pathophysiological importance. In contrast, the normotest is relatively insensitive to the presence of descarboxy coagulation factors in plasma. In umbilical cord plasma, inhibition of the thrombotest clotting time, defined as a difference of at least 5 seconds in the extrapolated "infinite high" concentrations in the so called thrombotest dilution curve (see figure 1) has been described in 67% of all normal full term healty newborns by van Doorm (9). This inhibition was not due to discrepancies between the individual coagulation factors II, VII and X or the presence of descarboxycoagulation factors. Another explanation for the prolonged thrombotest clotting times could be the presence of fibrin(ogen) degradation products. However, using a conventional method for the determination of fibrin(ogen) degradation products, van Doorm could not demonstrate the presence of these products in umbilical cord plasma samples (9). Several other investigators have also reported negative results, provided that the blood sampling and processing



THROMBOTEST DILUTIONCURVES:

① o—o = adult normal pool

② ■—■ = non-inhibitory cord plasma pool

③ □—□ = inhibitory cord plasma (1 Unit)

FIGURE 1

was adequate to avoid in vitro activation of fibrino(geno)lysis (10,11).
No sifnificant differences have been reported between the inhibited and non-inhibited group with regard to the levels of a number of protease inhibitors such as α_1-antitrypsin, α_2-macroglobulin, α_2-antiplasmin and antithrombin III. These results were based on immunological determinations (9).
Kirchhoff (12) found that the prolongation of the thrombotest clotting time was more pronounced in newborns delivered via difficult vaginal deliveries than in newborns delivered via Caesarean Sectio.
On the basis of experiments with protamine-neutralization and the fact that the action of the inhibitor appeared to be dependent on the presence of antithrombin III, van Doorm suggested that the inhibitor could be heparin-like. In this thesis (chapter I) we have described that in individual umbilical cord plasmas heparin-like activity is present only in ½-1% of all cases and therefore cannot be the explanation for the prolonging effect on the thrombotest clotting time, found in 67% of all newborns. Notwithstanding the possible complications in everyday practice, Thrombotest® and Normotest® are widely used as a screening test for the amount of the vitamin K-dependent coagulation factors present and by some authors even recommended as a diagnostic tool for a vitamin K deficiency in newborns (13).
The aim of this study was to assess the value of the thrombotest- and normotest clotting time in defining the levels of the vitamin K-dependent coagulation factors in newborns and to investigate whether these tests can indeed be used in clinical practice. In a previous study (14) we reevaluated the so called fetal fibrinogen and found that the properties of fetal plasma, attributed to fetal fibrinogen could be mimicked by adding purified fibrinogen fragment X to purified fetal and adult fibrinogen. No differences could be demonstrated between purified adult and fetal fibrinogen. We investigated the possibility that fibrin(ogen) degradation products could contribute to the prolongation of the thrombotest clotting time in newborns. We used new sensitive methods for the determination of fibrin(ogen) degradation products directly in plasma, i.e. enzyme immuno assays (EIA's) based on monoclonal antibodies directed against new antigenic sites, which become exposed upon the proteolytic breakdown of fibrin(ogen). These EIA's will be described elsewhere and are more sensitive than the assays currently available. The lower detection limit is less than 0.25 µg/ml.

Materials and methods

We investigated 40 umbilical cord plasmas of healthy full term newborns. No vitamin K had been administered to the mother or the newborns, nor any medication, known to influence the normal haemostatic mechanism. In all cases, the apgar score at 1' was more than 7 and the plasma pH above 7.2. Immediately after birth the umbilical cord was clamped and blood drawn by a

clean puncture of the umbilical vein. After discarding the first 5 ml, 9 volumes of blood were mixed (in a propylene tube) with 1 volume of an anticoagulant mixture (0.3 M hydroxyethylpiperazine ethane sulfonic acid (Hepes), 0.1 M disodium citrate dihydrate, 15 mM sodium azide and 1000 KIE trasylol/ml). This mixture effectively prevents in vitro fibrino(geno) lysis and in vitro activation of factor VII. Platelet poor plasma was obtained by centrifugation at 3000 x g for 15' minutes and rendered platelet free by centrifugation at 20 000 x g for 30' at 4 °C. The samples were frozen immediately at -80 °C in aliquots of 1 ml. Plasma of 10 adult donors were prepared in the same way and equal volumes mixed to serve as a 'control normal pool'. Thrombotest® and Normotest® reagents were obtained from Nyegaard, Oslo, Norway.

The assays were performed exactly as indicated in the manufacturers prescription. Thrombotest dilution curves were made by plotting the thrombotest clotting times (Y-axis) against the corresponding dilution of plasma (X-axis). These curves have been shown to be linear (15). Plasmas were diluted 1/2, 1/4, 1/6, 1/8 and 1/10 in Michaelis buffer (Na-veronal 0.15 M, Na-citrate 0.109 M, NaCl 0,15 M, pH 7.4) in plastic tubes immediately before they were tested and kept at room temperature. The Thrombotest® reagent was dissolved in 11 ml 3.2 mM $CaCl_2$. In each test tube 0.25 ml Thrombotest®-reagent was prewarmed at 37 °C. Clotting was initiated by adding 0.05 ml plasma dilution to the test tube. All measurements were performed in duplo within 30 minutes after the preparation of the dilutions.

At the intersection point in the Y-axis the concentration of the coagulation factors is "infinite high". In all cases the point of intersection on the Y-axis should be the same, provided that no inhibitors are present in the sample and that there are no major discrepancies between the levels of the individual vitamin K-dependent coagulation factors. If inhibiting material is present, the degree of inhibition can be expressed in units as indicated in figure 1. The slope of the curve is indicative for the amount of the coagulation factors II, VII and X present in the sample. The amount of factor II was determined with a one stage method with human thromboplastin and human factor II deficient plasma (16), using a computer program based on the clotting times of eight dilutions of adult normal pool plasma. Fibrin(ogen) degradation products were determined in serum using the conventional test kit of Wellcome and also directly in plasma using new enzyme immuno assays (EIA's) based on monoclonal antibodies. Details of the EIA's for the total fibrin (XDP) plus fibrinogen (FDP) degradation products (XPP plus FDP) and fibrinogen degradation products (FDP) separately will be published elsewhere (manuscripts submitted). For these two assays a monoclonal antibody, specific for degradation products of fibrin and fibrinogen was used as catching antibody. FDP was quantified with a horse-radish peroxydase (HRP) conjugate of a monoclonal antibody directed against fibrinopeptide A containing fibrinogen fragments as tag (Blood, in

press). The total of FDP and XDP was quantified with a HRP conjugate of polyclonal IgG's directed against fibrinogen, fibrin, X, Y, D, E and Aα, Bβ and γ chains. The fibrin degradation products (XPD) assay was carried out as described by Rylatt (17). Materials for the XDP assay were bought from Mablo, Brisbane, Australia.
Purified fibrin degradation products X (17) Y (18) and D-D (19) were prepared as described previously. They were added in small amounts to adult normal plasma and the non-inhibited pool of umbilical cord plasma.

Results

In table I the mean values, range and standard deviations are given of the thrombotest clotting times, the normotest clotting times and the one stage factor II (IIc) determinations. The IIc levels are expressed as a percentage compared with the adult normal plasma pool (100%). The clotting times are given in seconds and represent the values found when non diluted plasmas were used.

TABLE I

assay	N	mean value and SD	range
thrombotest clotting time	40	53.3" ± 8.1	35.2 - 66.5
normotest clotting time	40	32.8" ± 4.7	25.3 - 44.1
factor II (one stage)	40	50.6% ± 12.2	28.8 - 73.7

The correlation coefficient (r-value) between the thrombotest clotting time and the factor IIc levels was -0.46, between the normotest clotting time and the factor IIc levels -0.70, indicating that especially the thrombotest clotting time does not give reliable information on the level of the vitamin K-dependent coagulation factor II. This could be due to the fact that in 67% of all umbilical cord plasmas an inhibitor is found in thrombotest dilution curves (9). Fibrin(ogen) degradation products could not be demonstrated in any of the samples, using the test kit of Wellcome. The sensitivity of this method, however, is not very high. The detection limit is around 10 µg/ml, Moreover, some high molecular weight degradation products are thrombin clottable and will therefore not be measured in the conventional assay, which makes use of serum.
Recently (J. Koopman et al. and P.W. Koppert et al, manuscripts submitted) sensitive EIA's have been developed for the quantification of the total of fibrin plus fibrinogen degradation products (FDP + XDP), and of fibrinogen degradation products (FDP). The assay of XDP was performed as described by Rylatt (17). With these EIA's we determined the levels of FDP + XDP, FDP

and XDP in 6 inhibited and 6 non-inhbited umbilical cord plasma samples and adult normal pool plasma.
In the non-inhibited samples no fibrin(ogen) degradation products could be detected, whereas in the inhibited samples small amounts of fibrin(ogen) degradation products were present. These results are summarized in table II. The fibrin(ogen) degradation products are expressed as µg fibrinogen equivalents.

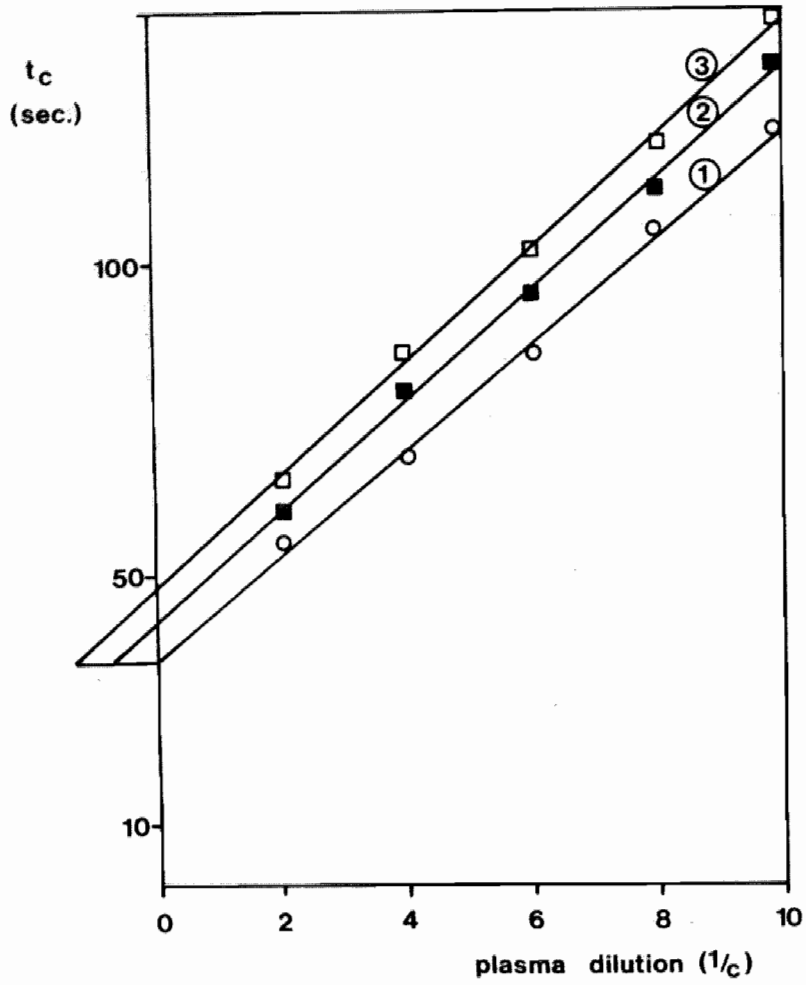
TABLE II

sample	degree of inhibition (U)	FDP + XDP	FDP	XDP
1	0.4	1.9	0.7	< 0.25
2	0.5	5.8	2.0	2.2
3	0.5	0.7	<0.25	< 0.25
4	1.3	12.8	3.7	4.6
5	0.3	2.2	0.5	< 0.25
6	0.3	1.8	<0.25	< 0.25
non inhibited cord plasma	0	< 0.25	<0.25	< 0.25
adult plasma	0	< 0.25	<0.25	< 0.25

In order to see, whether inhibition of the thrombotest dilution curve could be caused by degradation products, we added small amounts of purified degradation products X, Y and D-D to adult normal pool plasma and non-inhibited umbilical cord plasma.
The addition of fibrinogen fragment X to adult plasma even in relatively high concentrations gave only a slight prolongation of the thrombotest clotting time. In non-inhibited umbilical cord plasma, however, a more pronounced prolongation was found, at a final concentration of fragment X of 2.6 and 26 µg/ml. The results are given in figure 2 and 3. Addition of the fragments Y and D-D in these low concentrations did not affect the thrombotest clotting time of adult or non-inhibited umbilical cord plasma.

Discussion

Recently, the discussion in the literature on the prevalence of a vitamin K deficiency in newborns has regained much attention. A number of case reports has been published, showing evidence of a vitamin K deficiency, especially after the first few days of life (21,22). This deficiency has been attributed to changes in the food pattern (relative increase in breast fed infants since 1980) and a poorly developed colonization of the gut with vitamin K_2 producing bacteria (21).



① ○—○ = non-inhibitory cord plasma pool

② ■—■ = ① + 2.46 µg/ml fragment X

③ □—□ = ① + 24.6 µg/ml fragment X

THE EFFECT OF ADDING FRAGMENT $\overline{X}$ TO NON-INHIBITORY CORD PLASMA IN THE THROMBOTEST DILUTIONCURVE

FIGURE 2

The effect of adding fragment $\overline{X}$ to adult normal pool plasma in the thrombotest dilutioncurve

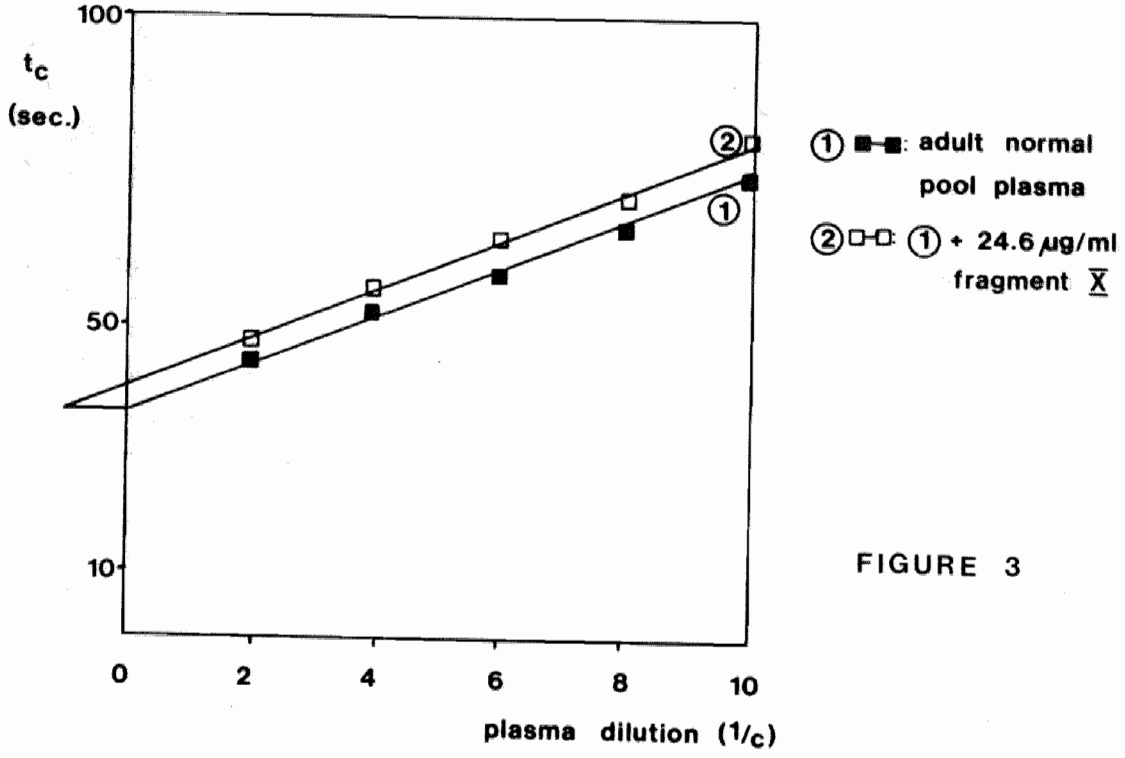


FIGURE 3

At birth, however, the vast majority of all normal newborns appears not to be vitamin K deficient (2,9), in spite of the fact that the vitamin K-dependent coagulation factors are around 50% of the values found in adults (1). In our opinion, the administration of vitamin K to newborns should be based on an adequate laboratory diagnosis of a vitamin K deficiency. This implies first of all adequate venapuncture and processing of the blood sample. In many routine clinical laboratories a prothrombin time test or one of its variants i.e. the thrombotest and normotest are used to assess the levels of the vitamin K-dependent coagulation factors. Several factors are known to cause a false prolongation of the prothrombin time and its variants. One of these is an excess of citrate in the case of an increased haematocrit (7).

Neonatal polycythemia is reported to occur in 10% of all newborns in the first week of life (23). In these cases a correction should be made for the amount of citrate used in the anticoagulant mixture in which the blood is collected.

In our umbilical cord samples the haematocrit values ranged from 0.44 - 0.54 and no haematocrit differences were observed between the inhibited and non-inhibited group. In this study, we have provided data to show that the thrombotest clotting time cannot be used as a reliable predictor to assess the vitamin K status of newborns. There was a poor correlation between the thrombotest clotting times and the prothrombin clotting activity (IIc) levels in umbilical cord plasma samples. This is probably due to an inhibitory factor of the thrombotest clotting time present in 67% of the cases.

Using sensitive EIA's, recently developed by the Gaubius Institute (Leiden, The Netherlands) we have demonstrated that fibrin(ogen) degradation products are present in umbilical cord plasmas, showing inhibition as judged from the thrombotest dilution curve, whereas they could not be detected in any of the samples of the non-inhibited group. Using conventional assays we were unable to detect these degradation products in umbilical cord blood. A number of investigators were unable to demonstrate fibrin(ogen) degradation products in serum prepared from umbilical cord blood (10,11). This can be due to the fact that the conventional method is not sensitive enough or to the possibility that high molecular weight fibrin(ogen) degradation products are partially thrombin clottable and will therefore not contribute to the amount of fibrinogen related antigen determined in the conventional method, since the latter assesses fibrinogen related antigen recovered in serum after complete clotting induced by thrombin and thromboplastin in the presence of trasylol.

The most striking finding was the presence of <u>fibrinogen</u> degradation products in our samples. This is only rarely found in disease states with primary fibrinogenolysis or with therapy with thrombolytic agents such as streptokinase. As we took care to minimize proteolysis in vitro, we feel that our data reflect an enhanced fibrino(geno)lytic activity in umbilical

cord blood in vivo. We were also able to induce the prolongation of the thrombotest clotting time by adding small amounts of purified fibrinogen fragment X to the non-inhibited umbilical cord plasma and adult normal pool plasma. The effect of the addition of fibrinogen fragment X appeared to be more pronounced with umbilical cord plasma than with adult plasma. We conclude that the prolongation of the thrombotest clotting time in newborns is only partially due to low levels of the vitamin K-dependent coagulation factors II, VII and X and that the results of this test should therefore not be used as evidence for a vitamin K deficiency. Too many factors, besides the levels of the vitamin K-dependent coagulation factors, influence the results of this screening coagulation assay. As shown here, also fibrinogen degradation products contribute to the prolongation of the thrombotest clotting time. It will be clear, that the limitation of the use of the thrombotest is even more pronounced, when the blood samples are drawn not carefully and when adequate inhibitors of in vitro proteolysis are lacking in the anticoagulant mixture in which the blood is collected. At the moment, it is unclear what the cause of the enhanced fibrino(geno)-lytic activity in normal newborns at birth is.

Fibrin(ogen)olytic activity is the result of the relative activities of tissue-plasminogen activator (tPA) and a fast-acting tPA inhibitor. Thus, increased fibrin(ogen)olytic activity may be the result of increased secretion of tPA from endothelial cells and/or a decreased level of tPA inhibitor. The latter may be caused by increased levels of activated protein C (APC), which is known to decrease the activity of tPA inhibitor (24).

The enhanced fibrin(ogen)olytic activity is probably one of the protective mechanisms against the development of thrombosis in the umbilical cord vessels.

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CHAPTER 4

THE SYNTHESIS OF PROTHROMBIN IN NEWBORN CALVES IN THE FIRST TEN DAYS OF LIFE

Summary

In this study, we describe the synthesis of the coagulation factors II (prothrombin) and VII in the first ten days of life in newborn calves. At birth the levels (coagulation activity) ranged from 35-65% of adult values. Within 3 days adult values were found, ranging from 70-110%. The levels of factor VII increased more rapidly than the levels of prothrombin. In none of the samples of normal calves descarboxyprothrombin could be demonstrated. This allows us to conclude that a vitamin K deficiency cannot be held responsible for the lower levels found in newborns. An additional argument against the vitamin K deficiency hypothesis is based on the finding, that the administration of abundant vitamin K_1 before delivery was not associated with higher values at birth or a more rapid increase after birth. The possibility, that newborns are unable to secrete descarboxy coagulation factors into the circulation in vitamin K deficiency states, was excluded by the fact that these products appeared in newborn plasma, upon the administration to the mother of low doses of the vitamin K antagonist phenprocoumon for two weeks before birth.
The fact, that the vitamin K-dependent coagulation factors at birth are around 50% of the adult values could partially be explained by an increased fetal plasma volume before birth (foetus plus placenta), assuming that this would imply an increased breakdown constant, and the normalization of this volume and breakdown constant after birth. In this way, the increase of the factors after birth could partially be explained.

Introduction

Brinkhous (1) was the first to demonstrate that the prothrombin levels in normal newborns are lower than in adults. Since then it has been well established that at birth all the vitamin K-dependent coagulation factors II, VII, IX and X and the anticoagulation factors Protein C and S are decreased to 30-60% of the normal adult levels (2). In 1894 Townsend (3) introduced the term "haemorrhagic disease of the newborn", a generalized bleeding syndrome in otherwise healthy infants, that occurred in the first week of life. This syndrome was attributed to a vitamin K deficiency soon after the discovery of this fat soluble vitamin in 1935 by Dam (4).
The administration of vitamin K was claimed to prevent this bleeding disorder (5,6,7,8). This could not be confirmed by other authors, who found no differences with regard to the incidence of bleeding problems between newborns who did and those who did not receive vitamin K prophylaxis at birth. However, in most studies higher prothrombin levels in plasma were reported in the group newborns that received vitamin K prophylaxis (9,10,11,12). It became clear that other factors could also be responsible for bleeding problems, such as consumption of fibrinogen, factor V and platelets and increased fibrinolysis. It is a well known observation that

disseminated intravascular coagulation and platelet consumption is a common disorder in the neonatal period (13).

If a vitamin K deficiency in normal newborns is present at birth it would be expected that descarboxy-coagulation factors can be demonstrated in umbilical cord plasma. This point is controversial in the literature. Some authors find no evidence for the presence of descarboxy-coagulation factors (14,15) whereas others demonstrate an altered prothrombin peak on crossed immunoelectrophoresis (16,17). The careful study of van Doorm et al. demonstrates beyond reasonable doubt that no descarboxy-prothrombin can be demonstrated in umbilical cord plasma in a normal Dutch (probably Western European) population of newborns (15). The absence of descarboxy-coagulation factors can theoretically also be due to the fact that newborns are unable to secrete these factors from the liver cell into the circulation. In man, only a few studies have been done (18,19) to evaluate the coagulation and fibrinolytic system in the first week of life of normal full-term infants. Many problems are encountered such as relatively large amounts of blood required and the difficulty in obtaining adequate material by a perfect venapuncture. In one of these studies (19), it was tried to solve these problems by using new micromethods and chromogenic substrates. None of these studies solves the problem of a possible vitamin K deficiency of the newborn. As it is ethically impossible to permit child birth in a woman that is known to be vitamin K deficient and as the Dutch health care system makes such an event infinitely improbable as a chance occurrence, we have therefore chosen an animal model. We studied the synthesis of prothrombin in the first ten days of life in normal newborn calves, measuring the prothrombin levels by clotting and immunological methods.

We also administered vitamin K_1 and the vitamin K antagonist phenprocoumon to the mother, to see whether the synthesis of prothrombin could be accelerated in the presence of abundant amounts of vitamin K and to see if the newborn calf is able to secrete descarboxy-coagulation factors into the circulation.

Material and methods

Blood samples were obtained from 30 normal healthy adult cows to serve as a normal pool for the bovine species. The blood was collected in 1 vol 0.1 M trisodium citrate dihydrate per 9 vol blood. Plasma was prepared by centrifugation at 2000 g for 15 minutes and rendered platelet free by a second run at 20.000 g for 30 minutes. Aliquots of 1 ml platelet free plasma were stored in plastic tubes at -70 °C until further use. Vitamin K_1 (Konakion®)(10 mg/ml) and phenprocoumon (Marcoumar®) were obtained from Hoffmann La Roche, Basle, Switzerland. Prothrombin levels were measured in 6 calves during the first 10 days of life after a normal pregnancy and birth à terme. To two pregnant cows vitamin K_1 was given for 2 weeks before delivery in a dosage of twice a day 5 mg intravenously.

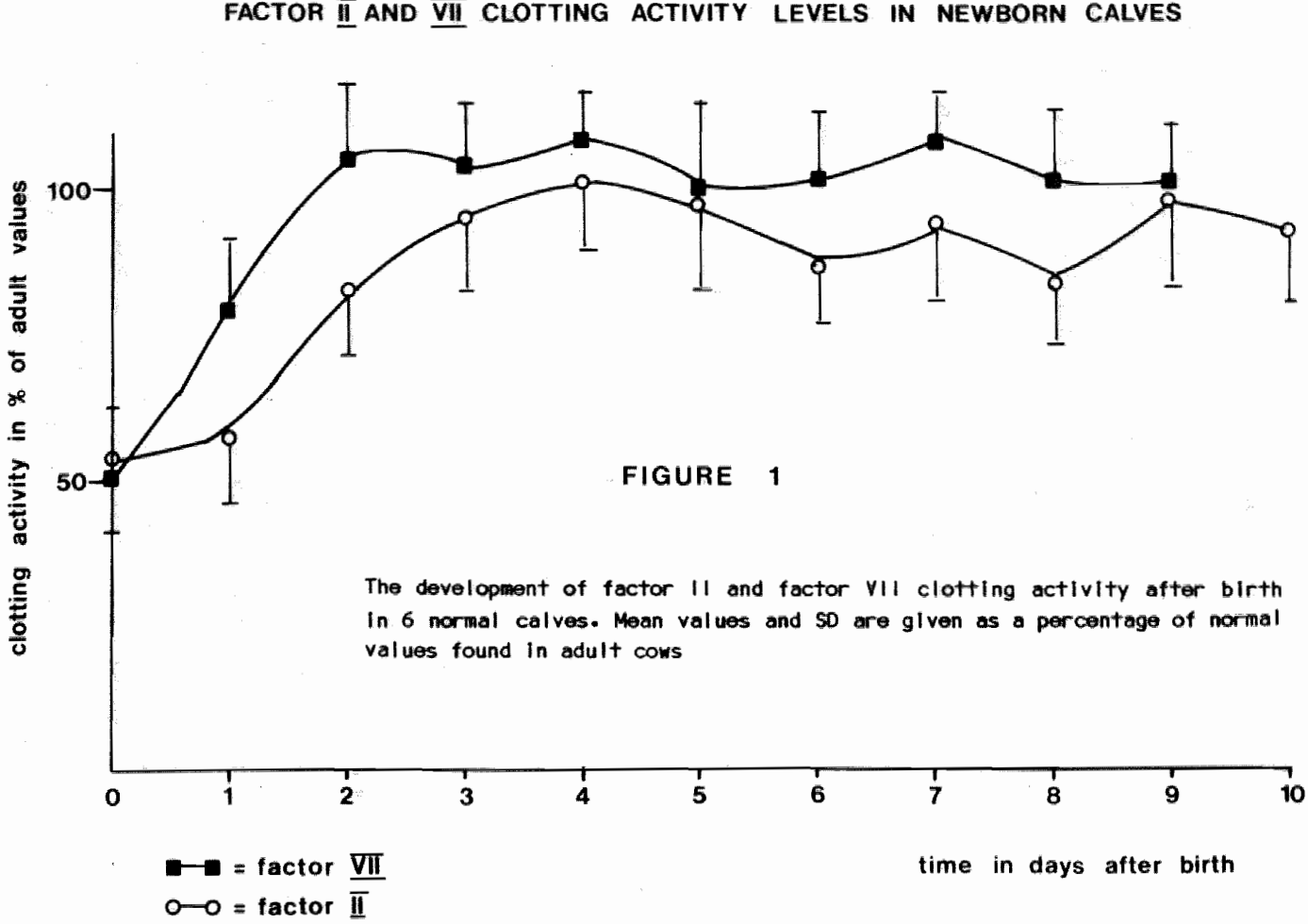
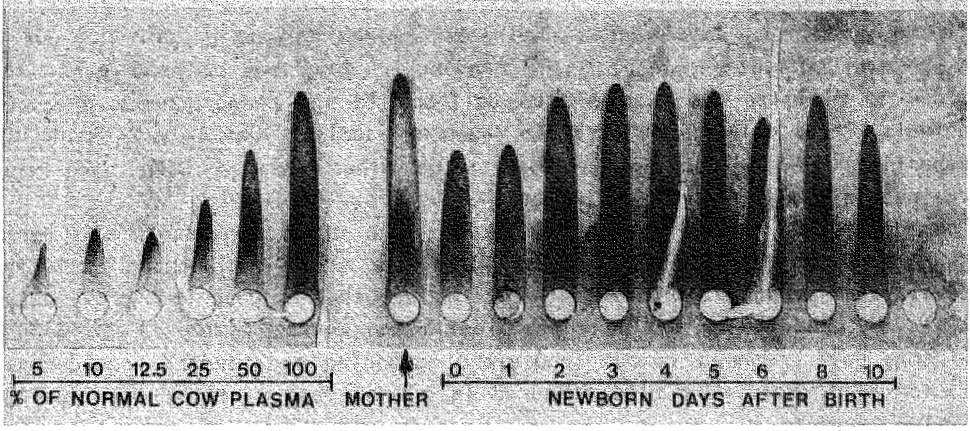


FIGURE 1

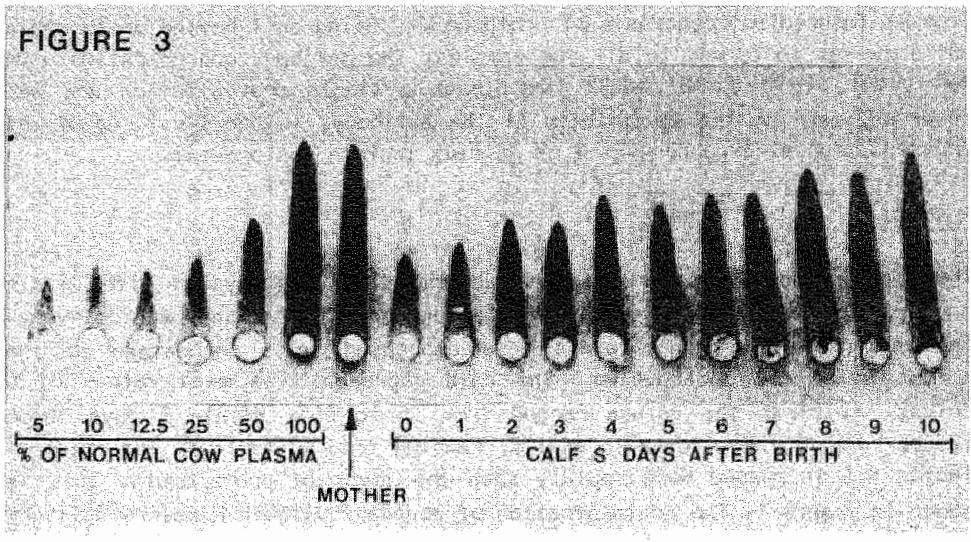
The development of factor II and factor VII clotting activity after birth in 6 normal calves. Mean values and SD are given as a percentage of normal values found in adult cows

FIGURE 2



The development of prothrombin antigen levels of one of the six normal calves after birth. (rocketimmunoelectrophoresis according to Laurell with a canine anti-bovine prothrombin antibody).

FIGURE 3



The development of the prothrombin antigen levels after birth in calf S.

Two other pregnant cows were given low doses of phenprocoumon intravenously (mean dose daily 5 mg) for 2 weeks before delivery to provide a steady state vitamin K deficiency with maternal prothrombin activity levels not lower than 70%.

Plasma vitamin K_1 levels were kindly determined for us by Dr. Thijssen, department of pharmacology, using previously described methods (17). In order to minimize the risk of the delivery in the phenprocoumon treated animals, these calves were born via Caesarean section (Dr. A. v.d. Bogaard, department of medical microbiology). The other animals were born via vaginal delivery. All deliveries were uneventful.

The blood from the calves was collected by a clean venapuncture of the jugular vein after discarding the first few ml's. Prothrombin activity levels were measured in an one stage clotting assay as described previously (18). We used bovine brain thromboplastin and bovine factor II deficient plasma, both prepared in our laboratory according to standard procedures. Factor VII levels were also measured in a one stage clotting assay using bovine reagents.

The percentage of prothrombin and factor VII clotting activities was calculated on the basis of dilutions of normal adult cow plasma in a computer program developed by our department (21). All measurements were performed in duplo. Prothrombin antigen levels were measured by rocket immunoelectrophoresis according to Laurell (22) using a canine anti-bovine prothrombin antibody.

Crossed immunoelectrophoresis of prothrombin, using anti-bovine prothrombin antibody was performed in all the samples. The antibody concentration used was 1 vol %. In normal adult cow plasma a single precipitation arc was observed, proving the specificity of the antibody. In the first run of the crossed immunoelectrophoresis 2 mM calcium lactate was present.

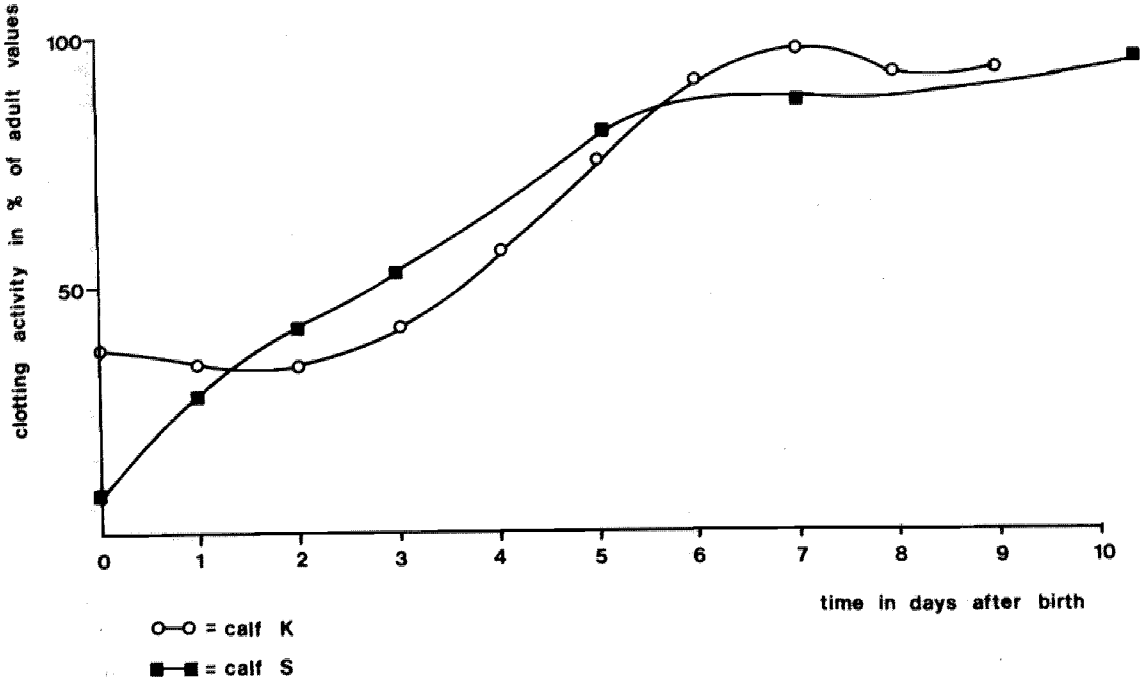
Results

The prothrombin levels were measured in the first ten days of life in six normal, full term calves, born after an uneventful pregnancy and delivery. The prothrombin activity levels were expressed as a percentage of the normal adult cow pool values. The data represent the mean values of 6 calves. At birth the values ranged from 35 to 65%. Within 3 days these values rose to those found in adults, ranging from 70 to 110%. The level of factor VII increased more rapidly than the level of prothrombin. This is shown in figure 1. The administration of abundant vitamin K before delivery was not associated with higher values at birth or a more rapid increase. The values found in these calves were in the same range as the values found in the normal calves. In the cows we found 21-25.8 ng vitamin K_1/ml plasma and in their offspring 4-6 ng vitamin K_1/ml plasma.

Figure 2 shows the factor II levels as determined by rocket immunoelectrophoresis in one of the six normal calves in the first ten days of life. We

FIGURE 4

THE DEVELOPMENT OF FACTOR II CLOTTING LEVELS IN CALVES K AND S



The development of factor II clotting activity levels in calves K and S. To calf S 10 mg vitamine K_1 (Konakion®) was given 4 and 24 hours after birth

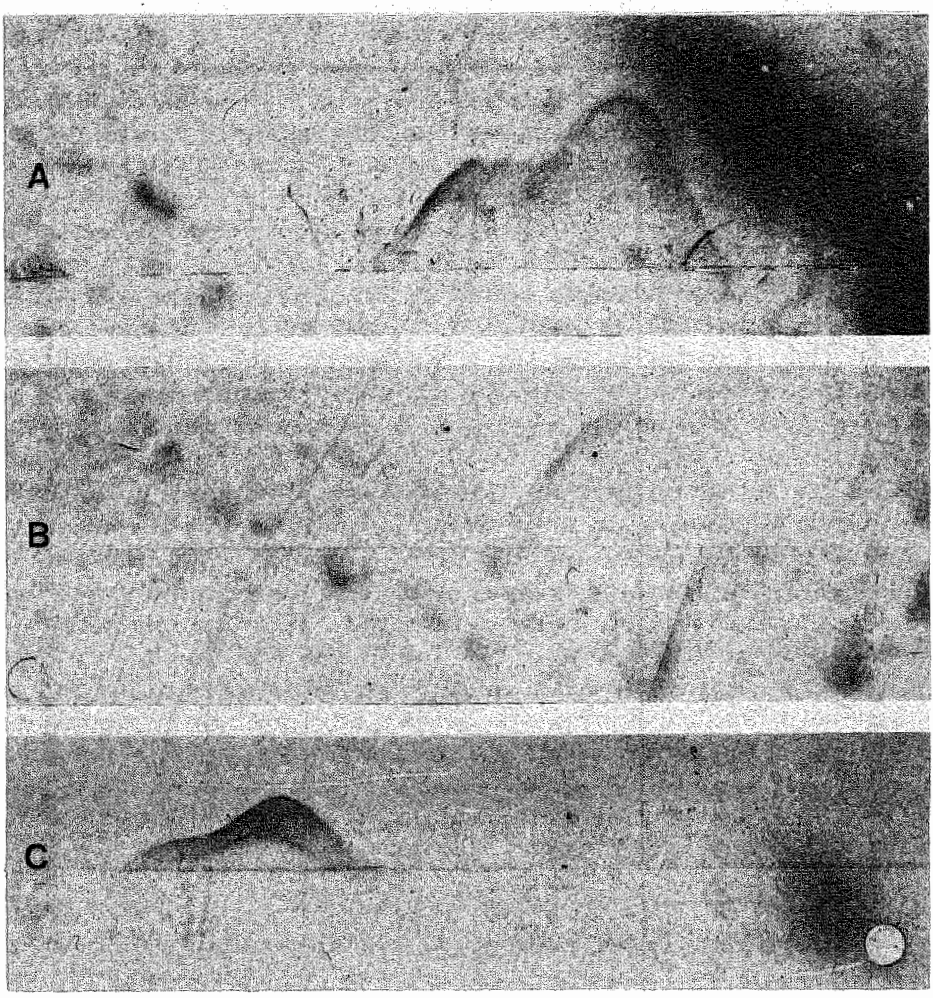


FIGURE 5

Crossed immunoelectrophoresis patterns against bovine anti-prothrombin antibody

A: 1:1 mixture of normal adult cow plasma and plasma of calf S obtained at birth

B: plasma of calf S obtained at birth

C: plasma of calf K obtained at birth

found no discrepancies between the prothrombin levels measured by the one stage method (clotting activity) and the levels determined immunologically by rocket immunoelectrophoresis. Crossed immunoelectrophoresis (CIE) of adult cow and newborn calf plasma showed a single precipitation arc at the same position (results not shown). We therefore concluded that no descarboxyprothrombin occurred in these plasmas.

Two pregnant cows were given low doses phenprocoumon during two weeks before delivery. The dosage was adjusted in order to keep the maternal prothrombin concentration between 70 and 80% of normal. In one of the pregnant cows (K) the phenprocoumon was stopped 48 hours before delivery. At birth the factor II concentration in calf K was 36% (clotting assay) and the ratio antigenic-biological level 1.4. In calf S the prothrombin concentration (clotting assay) was much lower: 8% and the ratio antigenic-biological level was 4.3.

In figure 3 the results are shown of the factor II antigen levels, determined by rocket immunoelectrophoresis of one of these two calves (calf S).

In figure 4 the development of the prothrombin clotting activity (one stage method) is shown of these two calves in the first 10 days of life. To calf S 10 mg vitamin K_1 (Konakion®) was administered 4 and 24 hours after birth, to calf K no vitamin K was given. In these calves a considerable delay was found in the synthesis of prothrombin, when compared with normal newborn calves.

In figure 5 crossed immunoelectrophoresis patterns are shown of plasma of calves K and S obtained immediately after birth. In calf K a double peak was observed (figure 5C). In plasma of calf S, at birth, almost all prothrombin related antigen migrated to the position of descarboxyprothrombin in the presence of calcium lactate (2 mM) in the first run (figure 5B). A mixture of normal adult cow plasma 1:1 with plasma of calf S, obtained at birth, showed a double peak (figure 5A).

Discussion

At birth, the levels of the vitamin K-dependent coagulation factors are around 50% of the normal values found in adults. The fact that the concentration of these proteins in plasma is lower in newborns has been accepted generally. Most studies have been performed in humans, but the same is also observed in a number of animals such as sheep, dog, rabbit and pig (23). This article as far as we know for the first time demonstrates the phenomenon in the cow.

The reason why the vitamin K-dependent coagulation factors are low at birth is unknown. It has been postulated that this is due to an "immaturity of the synthetic mechanism of the vitamin K-dependent coagulation factors in the newborn liver", with or without a vitamin K deficiency. Theoretically, the plasma level of a protein is determined by the equilibrium between its synthesis and its breakdown. The synthesis of the vitamin K-dependent

coagulation factors occurs in the liver. As is often the case in plasma proteins, the endproduct of the ribosomal protein synthesis in the hepatocyte, is not the final product. Vitamin K is required for the carboxylation of a specific sequence of Glu-residues at the NH_2-terminal end of the vitamin K-dependent coagulation factors to form Gla-residues.

In the case of a vitamin K deficiency uncarboxylated precursor molecules are secreted into the circulation, when the protein synthesis capacity of the liver exceeds the carboxylase system. In our study we found no evidence for a vitamin K deficiency in normal newborn calves, as we were unable to demonstrate the presence of descarboxy prothrombin in plasma: in all samples the prothrombin antigen levels paralleled the prothrombin clotting activity levels. In crossed immunoelectrophoresis patterns against bovine antiprothrombin a single precipitation arc at the normal position was found. This allows us to conclude that a vitamin K deficiency cannot be held responsible for the lower levels found in newborns.

According to Shearer, normal plasma vitamin K_1 levels in humans are in the range of 0.1-2 ng/ml plasma (24). We could confirm the results of Shearer (24) who found much lower vitamin K_1 levels in umbilical cord plasma in humans upon the administration of vitamin K_1 to the mothers before delivery. In our study in the bovine system, we administered abundant vitamin K_1 to the mother before delivery, which resulted in vitamin K_1 levels of 4-6 ng/ml in the plasma of their offspring, whereas in the corresponding maternal plasmas much higher values were found (21-25.8 ng/ml). These values are much higher than the normal plasma values and must therefore be regarded as more than sufficient for an optimal vitamin K-dependent carboxylation reaction. In these calves, the development of the prothrombin levels after birth, was in the same range as in the other calves. These results are an additional argument against the hypothesis that a vitamin K deficiency is responsible for the lower prothrombin levels in newborns.

The protein synthesis capacity of the newborn calf liver seems to be remarkably "mature". Adult values were reached within 4 days after birth, both for factor VII and prothrombin clotting activity levels. The factor VII levels increased more rapidly, presumably because of its shorter half life time. The increase of the vitamin K-dependent coagulation factors after birth could be due to the major changes in hepatic blood flow that occur after birth. As an alternative explanation, we can explain our results by the increased volume of the fetal plasma compartment (fetus plus placenta), assuming that this would imply an increased breakdown constant, and the normalization of this volume and breakdown constant after birth. In this way, the increase of the factors after birth could partially be explained. It may be questioned, if our results could be extrapolated to the human situation. The few results available at the moment indicate that also in man the factor VII levels reach adult values within one week, the prothrombin and factor X levels have been described to increase slowly and

to reach adult values in around 3 months, independent of the administration of vitamin K.

As mentioned above, the results of these studies can be criticized. Many problems are encountered, such as difficulties in obtaining adequate material and methodological problems.

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CHAPTER 5

THE PLACENTAL TRANSPORT OF [^{3}H]VITAMIN K_1 IN RATS

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Summary

In this paper we describe the placental transport of [^{3}H]vitamin K_1 in pregnant rats during the first 24 h after the oral administration of the vitamin. The total amount of label (expressed as dpm/mg protein) in the fetal liver increased from 1.1% (after 3 h) to 10.3% (after 24 h) of the values found in the corresponding maternal livers. The main part of the label in the fetal tissues consisted of water-soluble degradation products of vitamin K, however. Vitamin K_1, ranged from 0.24% (3 h) to 1.75% (24 h) of the values found in the corresponding maternal livers. In spite of the low placental transfer of vitamin K, we found no accumulation of coagulation factor precursors in the fetal rat liver microsomes. Moreover, we could not demonstrate any difference between adult and fetal rat liver microsomes with regard to the accumulation of endogenous substrate upon the administration of warfarin. From these results we conclude that a substantial placental barrier exists for the transport of pharmacological amounts of vitamin K_1 but that under physiological conditions sufficient vitamin K appears to be present in the fetal liver to ensure a full carboxylation reaction. Whether the low placental transfer rate of vitamin K_1 can be attributed to a dose dependent mechanism remains to be established. The carboxylase activity of adult and fetal rat liver microsomes was comparable, indicating that the newborn rat has an adequate carboxylating system.

Introduction

It has been well established that the concentrations of the fat-soluble vitamins in umbilical cord plasma are lower than those in maternal plasma (1-5). One of these vitamins is vitamin K, which is required for the synthesis of the coagulation factors II, VII, IX and X and the anti-coagulation factors Protein C and S. Using high performance liquid chromatography, Shearer et al. (1) demonstrated the vitamin K_1 concentrations in umbilical cord plasma to be much lower than those in maternal plasma. Additionally, a relatively small increase of vitamin K_1 was observed in the umbilical cord plasma after its administration to the mother, shortly before delivery. These results are in agreement with the assumption, that the placental transport of the fat-soluble vitamins occurs only to a very limited extent.

At birth the plasma concentrations of the vitamin K-dependent coagulation factors in full-term normal infants are decreased to levels of 30-60% of normal (6). These low levels have been attributed to a partial vitamin K-deficiency, but in that case the presence of descarboxy-coagulation factors (PIVKA's) in the umbilical cord plasma would be expected, and also a substantial difference between the coagulation factor levels in the plasma from newborns, who received vitamin K prophylactically at birth and

those who did not. Both points are rather controversial in the literature, however. Whereas Malia (7) and van Doorm (8) could not demonstrate des-carboxy-factors in umbilical cord plasma, the opposite is claimed by Muntean (9) and Corrigan (10). Secondly, it has not been shown convincingly that the administration of vitamin K_1 to normal newborns accelerates the increase of the plasma levels of the vitamin K-dependent coagulation factors (8). We have studied the placental transport of [^{3}H]vitamin K_1 in pregnant rats and its distribution over some maternal and fetal tissues at several time points after oral administration of the vitamin. Furthermore we have tried to find evidence for the occurrrence of a partial vitamin K-deficiency in the fetal rat livers. The results of these experiments are presented in this paper.

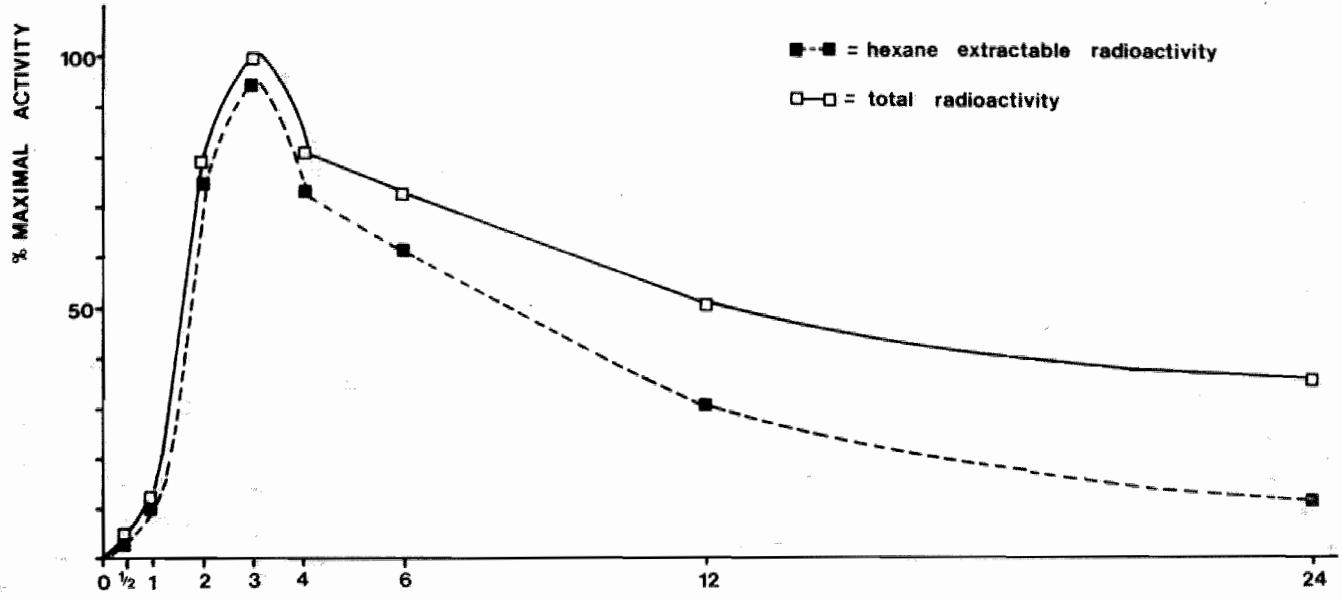
Experimental procedures

Materials. [^{3}H]vitamin K_1 (320 Ci/mol), which was a kind gift, as well as non-labeled vitamin K_1 (Konakion^R) were provided by Hoffmann-La Roche (Basle, Switzerland). The synthetic pentapeptide Phe-Leu-Glu-Glu-Leu (F L E E L) was obtained from Vega Biochemical Co. (Tucson, USA) and warfarin and dithiothreitol from Sigma (Saint Louis, USA). Protosol, Biofluor and Atomlight were from New England Nuclear (Dreieich, FRG). $NaH^{14}CO_3$ (56 Ci/mol) was purchased from Amersham (UK). All other chemicals were obtained from Merck (Darmstadt, FRG).

Methods. Pregnant, 12 weeks old rats (weights 240-270 gr) of the Lewis strain were used throughout our experiments. [^{3}H]vitamin K_1 (0.04 mCi) was administered orally in 0.5 ml sunflower oil, 24-48 h before the planned delivery. At the indicated times the animals were killed under ether anaesthesia and the peripheral blood was collected in heparinized plastic tubes after heart puncture. The various organs were removed immediately and washed with icecold buffer A (0.1 µM NaCl, 0.05 M Tris-HCl, pH 7.4). Peripheral blood samples (100 µl) as well as 200 mg pieces of the maternal and fetal livers and the placentae were digested in 2 ml Protosol and counted in 15 ml Biofluor in a Packard Tricarb scintillation counter. The results are expressed as dpm/mg protein or dpm/mg tissue (wet weight). The results of the peripheral blood samples are expressed as dpm/µl. In some experiments [^{3}H]Vitamin K_1 was extracted from the tissues in the following way. Tissue homogenates were prepared, 1/1 in buffer A in a tissue potter. To one ml of homogenate or peripheral blood 3 ml isopropanol containing 1 µg non-labeled vitamin K_1 were added. The mixture was shaken briefly on a whirlmixer and extracted two times with 2 ml hexane. The hexane fractions were collected and counted in Atomlight. The composition of the hexane extractable radioactivity was analysed by reversed phase thin layer chromatography as follows: The combined hexane fractions were evaporated to dryness in the dark under a gentle stream of oxygen free nitrogen at 40 °C.

FIGURE 1

[^{3}H]VITAMIN K_1 RADIOACTIVITY IN PERIPHERAL BLOOD



The appearance and disappearance of vitamin K_1 in the rat peripheral blood after a single oral dose of [H]vitamin K_1. For each time point 5 animals were used. The results are expressed as a percentage of the maximal radioactivity.

The residue was soluted in 50 µl of acetonitrile and 10 µl thereof was applied as a stripe (15 mm) onto the concentration zone of a Merck high performance RP18 thin layer plate (Merck, Darmstadt, FRG). The plate was briefly developed in acetonitrile until the solvent front reached the chromatographic layer. After evaporation of the acetonitrile the plate was developed in dichloromethane/acetonitrile containing 1% of water (3/7, v/v). Vitamin K_1 was visualized by illuminating the chromatogram at 366 nm for 5 min. A bright blue fluorescence emerges from vitamin K (Rf = 0 .12; Rf of vitamin K_1 epoxide = 0.20). The spot was scraped off and the radioactivity was counted by liquid scintillation. By this procedure it was found that the hexane extractable radioactivity consisted mainly (>90%) of vitamin K_1.

Microsomes from adult and fetal livers were prepared as described previously (12). The vitamin K-dependent carboxylation reaction was recorded by incubating the microsomes (1 mg of protein) in 0.25 ml reaction mixtures containing 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.4, 8 mM dithiothreitol, 1 M $(NH_4)_2SO_4$, 0.2 mM vitamin K hydroquinone, 10 µCi $NaH^{14}CO_3$, 12% (v/v) ethyleenglycol, 0.2% (w/v) CHAPS (3-[3-cholamidopropyl]dimethyl ammoniol-1-propane sulfonate) and 4 mM F L E E L as indicated. The mixtures were incubated in sealed tubes at 25 °C. The reaction was stopped with 2 ml 10% (w/v) trichloroacetic acid at the indicated times and non-bound $^{14}CO_2$ was removed by boiling for 2 minutes before the samples were counted in Atomlight. The amount of incorporation of $^{14}CO_2$ into endogenous substrate was measured in a similar way, except that the amount of microsomal protein was 2.5 mg and that no F L E E L and $(NH_4)_2SO_4$ were present in the reaction mixture. Protein concentrations were measured according to Lowry (11).

Results

The appearance and disappearance of vitamin K_1 in the rat peripheral blood was followed after a single oral dose of [^{3}H]vitamin K_1 to 40 pregnant rats. For each time point groups of 5 animals were used for obtaining blood and various organs. As is shown in figure 1, the maximal vitamin K concentration in blood is reached at 3 h and up till that moment degradation of vitamin K_1 is hardly observed. At later stages, the total label and the hexane extractable fraction thereof rapidly diverge, thus showing the degradation of the vitamin. The results are expressed as percentage of the maximal values of the total and the hexane extractable radioactivity.

The same rats were also used for the preparation of various organs: the maternal and fetal livers and the placentae. Again the radioactivity recovered in the whole tissues as well as that in the hexane-extractable fractions thereof were measured (see Table 1).

As compared to the maternal liver, only very low amounts of label accumulated in the fetal livers. Moreover it is obvious, that the main part of the label recovered from the fetal livers originates from water-soluble vitamin K-degradation products rather than from vitamin K itself. The total radioactivity found in the placentae appeared to be lower than the amount found in the peripheral blood samples of the mother at all time points (20-40%). From these results we concluded that (1) a considerable placental

TABLE I

The placental transfer of [H]-vitamin K_1

Label (dpm/mg protein)	Time(h) elapsed after start of experiment 3	12	24
Maternal	14287 ± 4445	28471 ± 3609	18536 ± 11033
Hexane extraction (%)	96.2 ± 0.7	91.8 ± 1.3	98.7 ± 1.3
Fetal liver	16.3 ± 34	1932 ± 214	2136 ± 764
Hexane extraction (%)	22+1 ± 2	16.5 ± 0.9	16+8 ± 1.9
Ratio fetal/maternal			
in liver tissue:	0.011	0.063	0.103
in hexane extracts:	0.0024	0.010	0.017
Label in placentae tissue (dpm/mg wet weight)	131 ± 58	149 ± 55	135 ± 50

The distribution of [^{3}H]-Vitamin K_1 in maternal and fetal tissues. The data are the means of 5(maternal tissues) and 20(fetal tissues) animals ± the standard deviation at each indicated time point. The protein content of fetal liver was about 50% of the maternal liver

barrier exists for the transport of vitamin K_1 and (2) that this barrier seems to be lower or absent for the water-soluble vitamin K-degradation products. Since we were interested whether the impeded vitamin K transport leads to a partial vitamin K-deficiency in the fetal liver, we have prepared the microsomal fractions thereof and investigated a) the levels of endogenous substrates (e.g. carboxylatable precursors of coagulation factors) for carboxylase and b) the amount of hepatic carboxylase. In the

case of a vitamin K-deficiency an accumulation of endogenous substrate is to be expected (12). For this purpose the fetal livers were compared with the corresponding maternal livers. No differences were observed between the newborn and adult rat liver microsomes with regard to the accumulation of endogenous substrate (see figure 2). We found around the same (low) values in non pregnant female rats and three days old rats (results not shown). The amount of carboxylase was determined in the presence of an excess of the synthetic substrate F L E E L and as can be seen from table 2 no significant differences were observed.

TABLE II

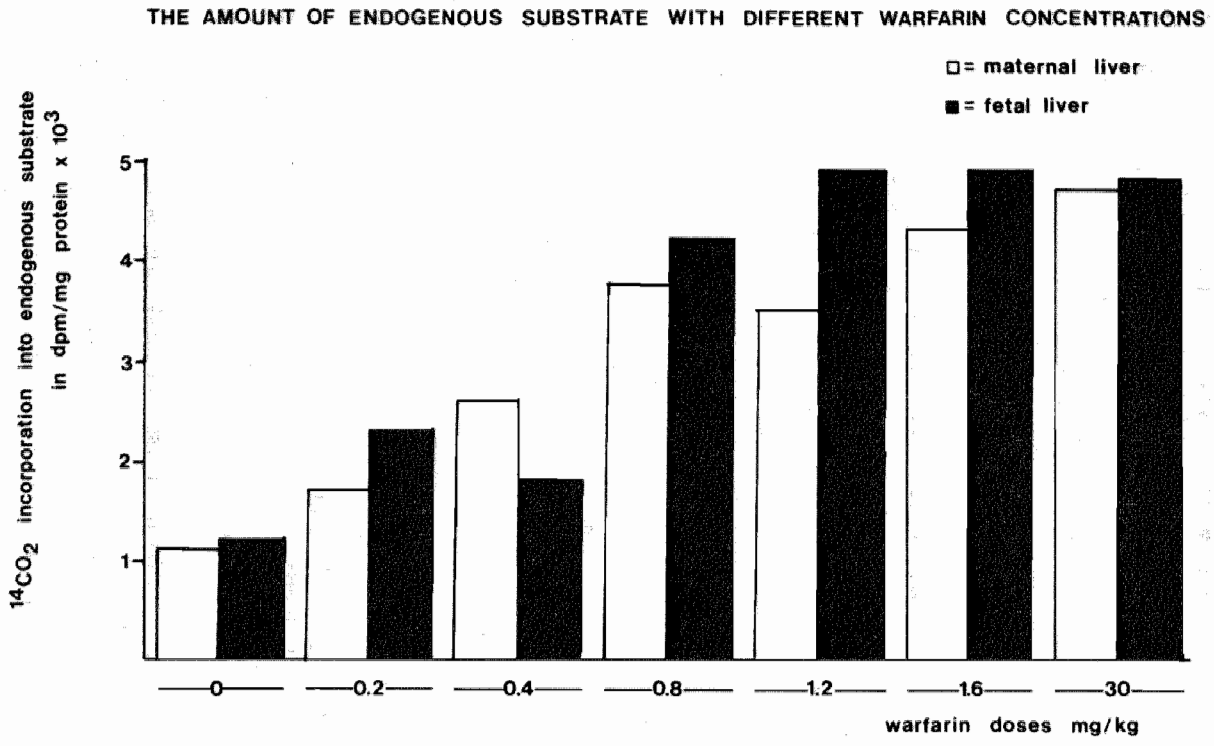
The vitamin K-dependent hepatic carboxylase activity

The vitamin K-dependent carboxylase activity was measured as indicated in METHODS, in the presence of an excess of exogenous substrate (F L E E L). The reaction was linear in the first 30 minutes. The results are given in dpm/mg protein/min.

Maternal liver (n=6)	1254 ± 178
Fetal liver (n=5)	955 ± 106

This indicates that the carboxylating enzyme system in the fetus is comparable with the adult carboxylating system and therefore capable of maintaining a normal carboxylation rate. Since the amounts of endogenous substrate were low in all cases, no evidence could be obtained for a relevant deficiency of vitamin K in the fetal liver. Although the supply of vitamin K in fetal liver thus seems to be sufficient for the normal functioning of carboxylase, it still could be that this supply is marginal and that small perturbations have a more profound effect in the fetal than in the maternal liver. Therefore, we measured the accumulation of endogenous substrate for carboxylase in maternal and in fetal liver after the subcutaneous administration of different doses of warfarin to pregnant rats, two days before the planned delivery. The animals were killed 24 hours after the warfarin treatment and the microsomal fractions were prepared from the maternal and fetal livers. The amount of endogenous substrate was determined in the absence of F L E E L and expressed as dpm/mg protein. The results are given in figure 2 and demonstrate that the accumulation of endogenous substrate for carboxylase in maternal and fetal liver upon the administration of warfarin was comparable.

FIGURE 2


THE AMOUNT OF ENDOGENOUS SUBSTRATE WITH DIFFERENT WARFARIN CONCENTRATIONS
□= maternal liver
■= fetal liver
$^{14}CO_2$ incorporation into endogenous substrate in dpm/mg protein x 10^3
5
4
3
2
1
0
0.2
0.4
0.8
1.2
1.6
30
warfarin doses mg/kg

Discussion

It is generally assumed, that the placental transport of the fat-soluble vitamins A, D, E, and K resembles that of lipids. In blood plasma the vitamins are bound to proteins and lipoprotein complexes. The way in which the vitamins are subsequently released to the placental membranes is not yet clear, whereas the transfer from the maternal to the fetal side of the placenta is thought to occur by simple diffusion (5). Finally, (lipo-) proteins again mediate in the plasma transport of the vitamins to their fetal target organs. The complexity of this transport mechanism may be the cause of the limitations, which are set to the passage of fats and fat-soluble vitamins through the placenta. The first indication for the existence of a placental barrier for vitamin K was reported by Shearer et al. (1), who demonstrated that the vitamin K concentration in umbilical cord plasma is lower than that in maternal plasma. Since these authors worked with human volunteers, the actual vitamin K concentrations in the liver could not be determined, however. The low amounts of vitamin K in umbilical cord plasma might also be explained, for instance, by assuming that the relatively big liver and the rapid growing bones of the fetus extract vitamin K from plasma more efficiently than the corresponding maternal tissues do. The relatively low levels of the (lipo)proteins in newborn plasma offer another possible explanation, providing a decreased possibility for the transport of the vitamin. Using pregnant rats as an experimental animal system, we have demonstrated in this paper that after the oral administration of vitamin K_1 to the mother, the uptake of the vitamin (expressed per mg of protein) by the fetal liver is 1-2% from that of the maternal liver. Obviously this figure is even far lower when the uptake is expressed per whole organ. Our results may also be slightly influenced by the vitamin K_1 present in the blood volume of the tissues. For adult rat liver this is supposed to be 0.26 µl/mg (13), for fetal liver it is probably somewhat higher. Correction of our data for the tissue blood volume does not lead to different conclusions, however. The fetal livers contained relatively high amounts of water-soluble vitamin K degradation products, indicating that the placental barrier for these compounds is low or absent.

Since apparently the placental transport of vitamin K is hampered, it is to be expected that the vitamin K level in the fetal liver is low. In the case of a vitamin K deficiency the accumulation of endogenous substrate for carboxylase (coagulation factor precursors) in the hepatic microsomal fraction is to expected (12). At later stages also non-carboxylated coagulation factors can be detected in blood plasma. We have been unable to detect increased amounts of carboxylatable proteins in the fetal livers. Therefore, no evidence could be obtained for a relevant vitamin K deficiency in fetal liver, assuming that the synthesis of carboxylatable proteins in fetal liver is comparable with that in adults. Moreover, in

vivo experiments in which pregnant rats were treated for a short time with different doses of warfarin, did not reveal a relatively greater increase in the accumulation of endogenous substrate in the fetal livers. It must be concluded therefore, that in the fetal liver probably sufficient vitamin K is present for a complete carboxylation reaction. Apparently this conclusion is in contradiction with the observed placental barrier for vitamin K_1, but our results may be explained by assuming that the placental transfer of vitamin K is bound to a maximum. Physiological concentrations of the vitamin may then be transported without difficulties, but during experiments in which the far higher pharmacological doses of [^{3}H]vitamin K_1 were administered to the animals, the placental transport did not increase proportionally. It would thus be interesting to measure directly the levels of endogenous vitamin K in normal maternal and fetal liver, but up till now the determination of physiological concentrations of vitamin K in tissues has proven to be a difficult task. For technical reasons it was also not possible to simulate in this set of experiments the physiological situation obtained via long-term administration of physiological (low) doses of vitamin K.

We conclude that, in spite of the low placental transfer rate of vitamin K_1, no vitamin K deficiency can be demonstrated in newborn rats under normal conditions. Under the assumption that the same holds for human newborns, it may be questioned, if the administration of vitamin K to normal newborns at birth is indicated. However, it will be clear, that in the case of a vitamin K deficiency in the mother and/or long term treatment during pregnancy with vitamin K-antagonists or anticonvulsant drugs, the fetus will be relatively more at risk than the mother for bleeding complications. In that case, repeated pharmacological (high) doses of vitamin K are required for a normal carboxylation reaction in the fetal liver, one dose being not sufficient.

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CHAPTER 6

A NEW METHOD TO ASSESS THE AMOUNT OF VITAMIN K IN LIVER MICROSOMES

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Summary

In this paper, we describe a new method for the determination of the amount of vitamin K present in the microsomal fractions of rat- and bovine liver tissue. This method is based on the biological activity of vitamin K in its function as a coenzyme for the carboxylating enzyme system. The amount of vitamin K present in the microsomal fractions is relevant for its biological activity. The values are expressed as ng phylloquinone equivalents per mg microsomal protein.
In adult rat- and cow liver the values ranged from 1.6-4 ng per mg microsomal protein. Upon the administration of 10 mg vitamin K_1 a 4-5 fold increase of the values was found in rats. In a preliminary experiment we found lower values (around 50%) in newborn rat liver microsomes.

Introduction

It has been well established that the natural fat-soluble vitamin K compounds are involved in a posttranslational step in the synthesis of a number of proteins (1). This process leads to the formation of gamma-carboxyglutamic (Gla) residues. Besides vitamin K, a carboxylating enzyme system (carboxylase) is required for this posttranslational modification (1). The physiological significance of the Gla-residues is related with their binding of calcium. Gla-residues were discovered about ten years ago in the coagulation factor prothrombin (2). Prothrombin, as well as most other coagulation proteins, is synthesized in the liver. It is not generally known, that vitamin K-dependent carboxylase is also present in other tissues such as lung, kidney, bone, spleen and testis (3). Moreover, numerous Gla-containing proteins have been detected such as bone Gla protein (osteocalcin) and renal Gla protein. In most cases, however, their function is not wholly understood. Compared with the other fat-soluble vitamins A,D and E, relatively few data are available on the physiological concentrations of vitamin K present in biological samples. This is due to the very low concentrations of vitamin K in body tissues and fluids and a number of technical problems.
Until 1970, vitamin K concentrations were measured in the chick bioassay (4). In this assay, chicks were fed with a diet low in vitamin K, which resulted in a prolongation of the prothrombin clotting time. The shortening of this clotting time upon the addition of the sample to the diet was measured and compared with the effect of adding phylloquinone (vitamin K_1) to the diet. This method appeared to be rather sensitive (200 ng/gram of diet), but inaccurate and time consuming. Physicochemical methods were also unreliable. Some improvement came, when chromatographical methods were introduced, such as column liquid chromatography, thin layer chromatography (TLC) and gaschromatography. The development of high performance liquid chromatography (HPLC) provided a major improvement. Using this method, it

became also possible to measure vitamin K_1 levels in human plasma (5,6). Modifications of the HPLC method have recently been introduced which have further improved the efficiency and reproducability. In this method the detection limit for vitamin K_1 and vitamin K_1-epoxide is as low as 25 pg/ml (7). However, also the very sensitive HPLC methods have the disadvantage of not reflecting biological availability of vitamin K. Therefore, we developed a new bioassay for the determination of the amount of vitamin K present in liver microsomes. This method is based on the biological activity of vitamin K in its function as coenzyme for the carboxylating enzyme system, which is localized in the microsomal fractions.
In the bioassay phylloquinone (vitamin K_1) as well as the menaquinones (K_2-vitamins) are measured. Both forms of vitamin K are known to be biologically active, although the role of the K_2-vitamins in the carboxylation reaction is less well investigated than that of phylloquinone.

Experimental procedures

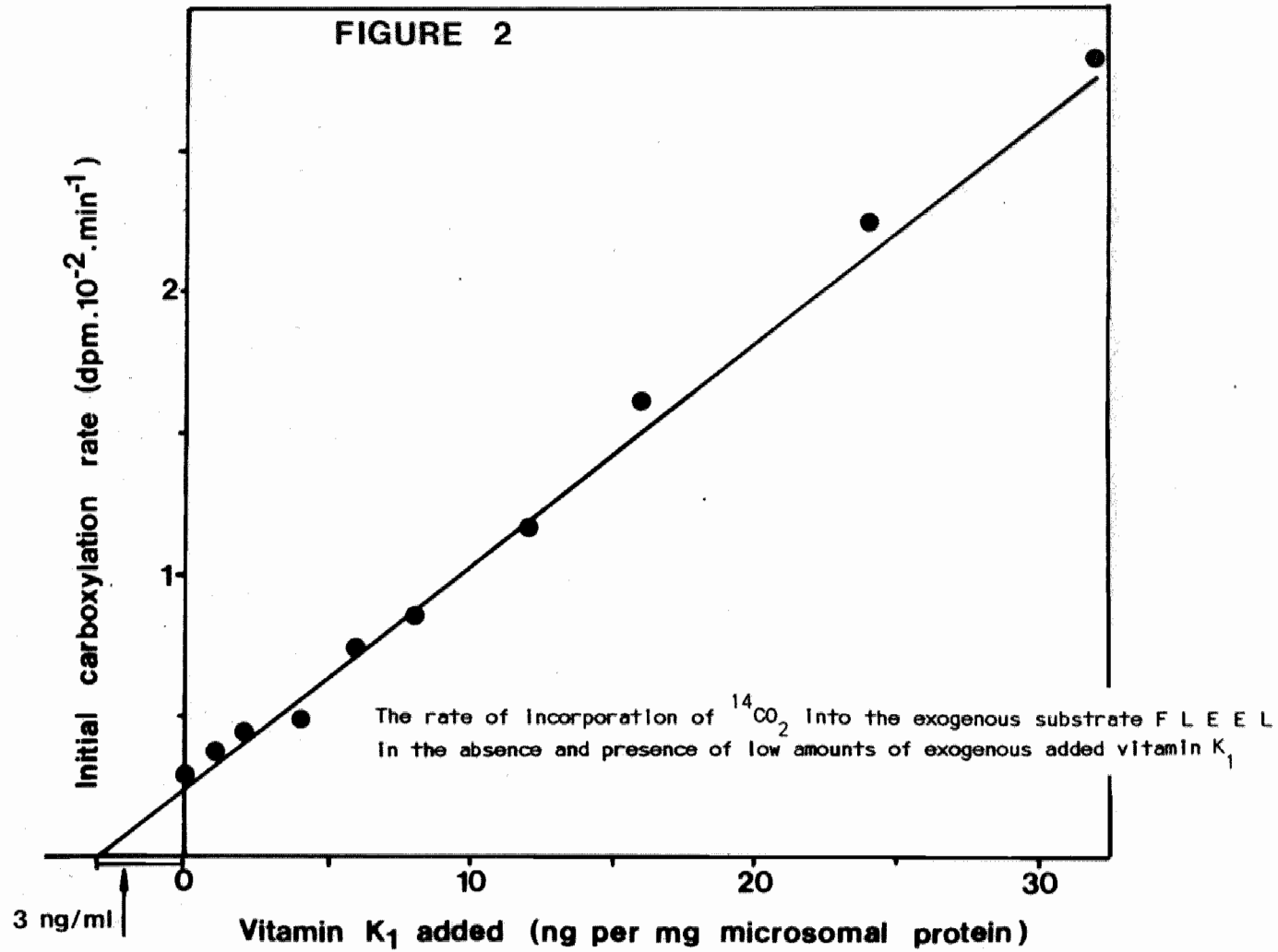
Materials

Vitamin K_1 (Konakion^R) was obtained from Hoffmann-La Roche (Basle, Switzerland). CHAPS (3-[(3-cholamidopropyl-dimethylammonio]-propane sulfonate) and DTT (dithiothreitol) were from Sigma (St. Louis, USA) and the synthetic pentapeptide Phe-Leu-Glu-Glu-Leu (F L E E L) was obtained from Vega Biochemicals (Tucson, USA). $NaH^{14}CO_3$ (56 Ci/mol) was purchased from Amershan (U.K.) and Atomlight from New England Nuclear (Dreieich, F.R.G.). All other chemicals were from Merck (Darmstadt, FRG).
Female Lewis rats of 12 weeks old (weights 220-250 gram) were used throughout our experiments. In one set of experiments pregnant 12 weeks old Lewis rats were used, as well as their offspring immediately after birth. All animals were fed with RMN-B, 12-15 gram dry matter per day obtained from Hope Farms (Woerden, The Netherlands). According to the manufacturer the vitamin K content of this preparation is 10.5 mg/kg. Fresh cow livers were obtained from the slaughterhouse.

Methods

The microsomal fractions of the tissues were prepared in a similar way as described earlier (3). After three washing cycles, the microsomes were suspended in buffer A, containing 0.1 M NaCl, 0.05 Tris-HCl, pH 7.4, 20% (v/v) ethyleneglycol. The samples were stored at -70°. Protein concentrations of the microsomal fractions were measured according to Lowry (8). The carboxylase activity was measured by incubating 0.25 ml reaction mixtures at 25 °C in sealed tubes containing 0.5 - 1 mg microsomal protein, 0.2% (w/v) CHAPS, 4 mm F L E E L, 8 mM DTT, 0.01 mCi $NaH^{14}CO_3$ and 1 M $(NH_4)_2SO_4$. The reaction was stopped at 20 minutes by adding 2 ml 5% trichloroacetic acid and the non-bound $^{14}CO_2$ was removed by boiling the solution for two minutes. The samples were supplemented with 10 ml Atomlight and counted in a Beckman scintillation counter. The carboxylase-

FIGURE 2



The rate of incorporation of $^{14}CO_2$ into the exogenous substrate F L E E L in the absence and presence of low amounts of exogenous added vitamin K_1

activity was expressed as $^{14}CO_2$ (dpm) incorporated in F L E E L per minute. The blank values were subtracted. Similar samples were incubated in the presence of low amounts of exogenous added vitamin K_1 in a range from 1 -16 ng. Vitamin K_1 was diluted in buffer A immediately before starting the incubation to minimize the light induced degradation of vitamin K. For each sample freshly prepared vitamin K_1 dilutions were used. The rate of $^{14}CO_2$ incorporation was plotted against the concentrations of vitamin K_1 added. This curve was linear in the range of vitamin K_1 concentrations used. From these curves the amount of vitamin K present in the sample can be calculated and expressed as ng vitamin K_1 equivalents per mg microsomal protein. To 5 adult female rats 10 mg vitamin K_1 were administered one hour before sacrifice. Protein concentrations were determined according to Lowry (8).

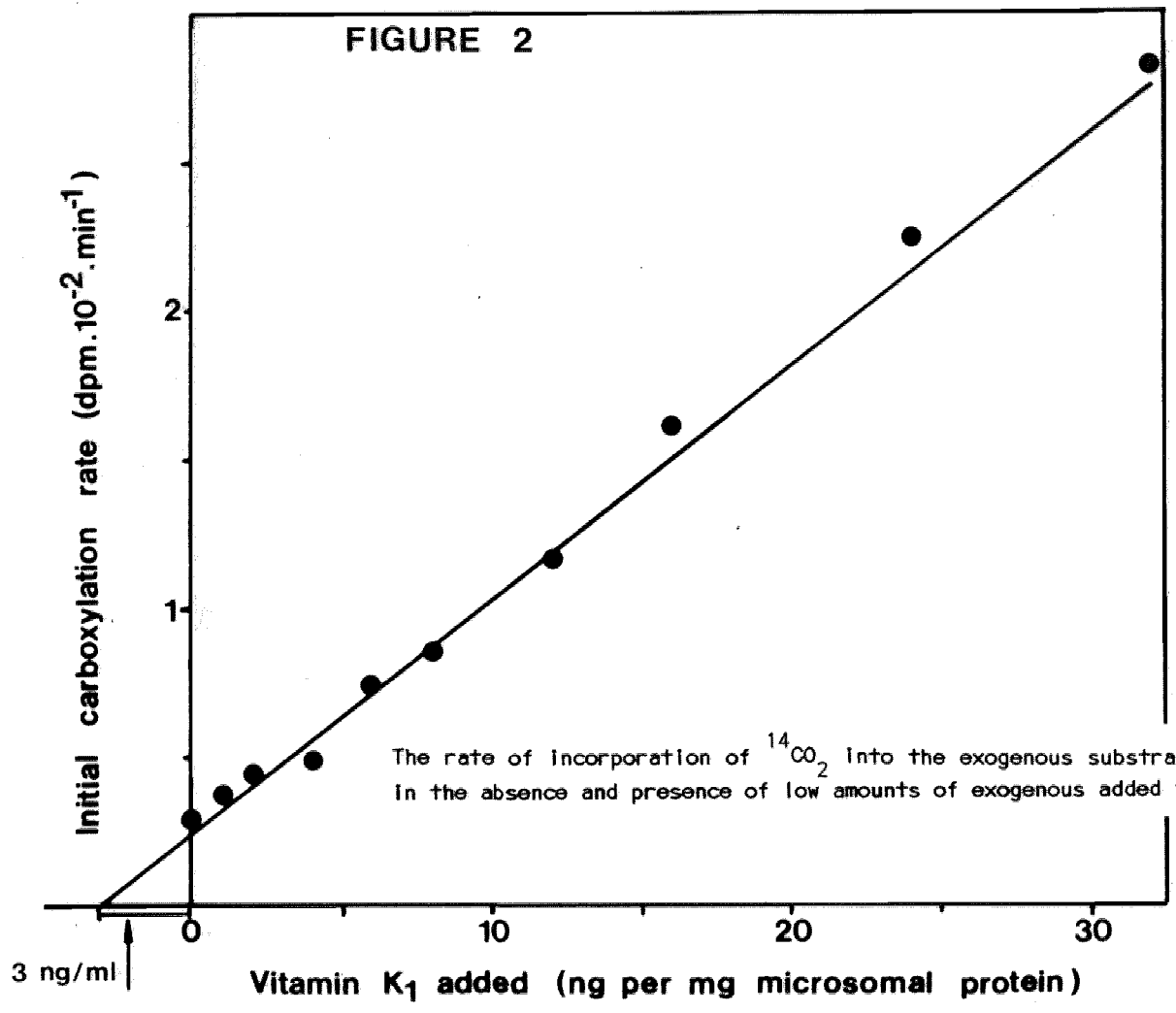
Results

In order to define optimal protein concentrations for our experiments we determined the relation between the protein concentration in the microsomal fractions, determined according to Lowry (8), and F L E E L carboxylation. This curve appeared to be sigmoideal, as can be seen in figure 1. In our reaction mixtures protein concentrations were used, which are in the linear part of the sigmoideal curve. In figure 2 the rate of incorporation of $^{14}CO_2$ into the exogenous substrate F L E E L is plotted against the concentration of vitamin K_1 added. Assuming, that the interception point on the ordinate in this curve represents the rate of incorporation determined by the amount of vitamin K present in the microsomes, this amount can be calculated by extrapolating the linear curve as indicated in figure 2. In this case the amount of vitamin K was 3 ng phylloquinone equivalent per mg microsomal protein. Using different protein concentrations in the linear part of the sigmoideal curve, comparable results were obtained. This is shown in figure 3.
In adult rat liver microsomes the values ranged from 1,6 - 4 ng per mg microsomal protein (N=4). Comparable values were found in cow liver microsomes. In the vitamin K_1 treated animals 4-5 fold higher values were found. In a pilot experiment the vitamin K concentration was also determined in newborn rat liver microsomes. The figures were distinctly lower (50%) than those obtained in maternal rat liver microsomes. Further research concerning this point is in current progess in our laboratory.

Discussion

In the literature remarkable differences are reported with regard to the amount of vitamin K in rat liver (9,10). The values obtained by the chick bioassay were much higher than those obtained by the application of high-performance liquid chromatography. Vitamin K can be present in

FIGURE 2



The rate of incorporation of $^{14}CO_2$ into the exogenous substrate F L E E L in the absence and presence of low amounts of exogenous added vitamin K_1

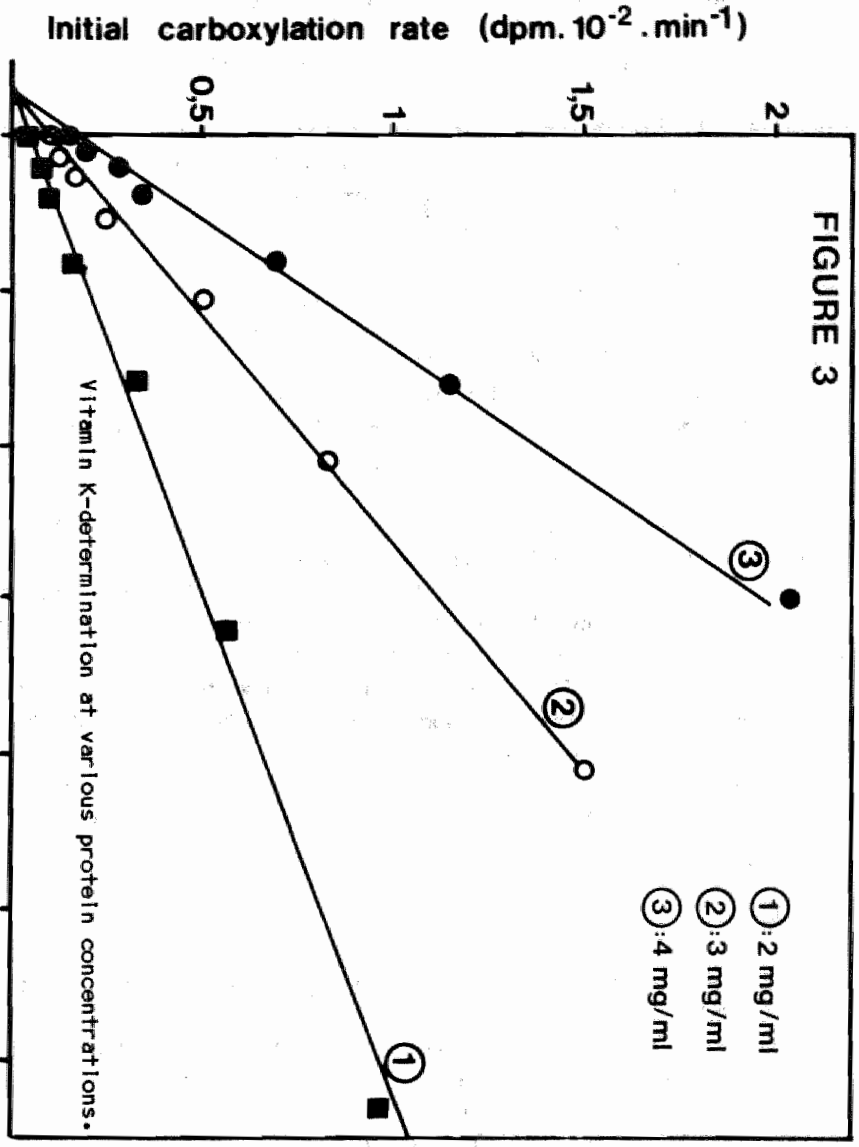

Initial carboxylation rate (dpm. 10^{-2} . min^{-1})
2
1,5
1
0,5
0
20
40
60
Vitamin K_1 added (ng/mg microsomal protein)
① : 2 mg/ml
② : 3 mg/ml
③ : 4 mg/ml
Vitamin K-determination at various protein concentrations.

FIGURE 3

membranes and other (phospho)lipids. However, only the vitamin K available for the carboxylating enzyme system, which is present in microsomes, is relevant. Therefore, we developed a new bioassay for the amount of vitamin K present in microsomes. The data obtained were dependent upon the intake of exogenous vitamin K. After the administration of 10 mg vitamin K_1 a 4-5 fold increase was found.

It is difficult to compare the values obtained with our bioassay with those found by Haroon using a HPLC assay. In the bioassay phylloquinone (vitamin K_1) as well as the menaquinones (K_2-vitamins) are measured, whereas in the HPLC assay only vitamin K_1 is measured. The role of the K_2-vitamins in the carboxylation reaction is less thoroughly investigated than that of phylloquinone. In one report (10) it is suggested, that on a molar basis some of the K_2-vitamins are considerably more active than vitamin K_1. Another important difference is the fact that in the HPLC method extracts are prepared of whole liver tissue, whereas in our bioassay microsomes are used. At the moment it is unknown how vitamin K is distributed over the several lipid components of the liver cell. It would also be of interest to know if the distribution of vitamin K in the liver cell is different during vitamin K deficiency and what is the minimum requirement of vitamin K needed for full carboxylation. We have also studied a few newborn rat liver microsomal fractions. There are at least 2 reasons, why lower values are to be expected in newborns. First, the placental transport of vitamin K_1 is low (11). Secondly, it may be that the distribution of vitamin K in the various organs differs from that in adults, since for instance the rapidly growing newborn bone tissue consumes high amounts of vitamin K for the carboxylation of osteocalcin.

In a preliminary experiment we found indeed lower vitamin K concentrations (50%). These data must be confirmed in a larger group of animals, before they can be regarded as conclusive.

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CHAPTER 7

REEVALUATION OF SOME PROPERTIES OF FIBRINOGEN, PURIFIED FROM CORD BLOOD OF NORMAL NEWBORNS

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Summary

In this study we compared some properties of fibrinogens, obtained from normal adult and umbilical cord plasma. Fibrinogen preparations were made under conditions, which minimize proteolytic breakdown in vitro. We were not able to demonstrate any differences between both purified fibrinogens as to the effects of pH and ionic strength on its clotting properties, the K_m for thrombin, SDS polyacrylamide gelelectrophoresis behaviour or carbohydrate content.

However, the phosphorus content of cord fibrinogen was 3-4 times higher than that of adult fibrinogen.

The accelerating effect of calcium on the thrombin clotting time was more pronounced for newborn cord plasma and for purified cord fibrinogen preparations as compared with adult fibrinogen. This might be explained by the higher phosphorus content of the cord fibrinogen molecule.

The thrombin clotting time of both purified adult and cord fibrinogen was markedly prolonged, when increasing amounts of fibrinogen degradation product fragment X were added to the fibrinogen solutions under conditions with high pH or high ionic strength.

At high pH the effect of adding fragment X was more pronounced in cord fibrinogen preparations. Therefore, mixtures of purified fibrinogen and fragment X have several properties in common with fetal fibrinogen.

These observations show, that some of the properties that have been attributed in the literature to a distinct fetal fibrinogen can be caused by the presence of fragment X in the cord fibrinogen preparations.

Introduction

The possible existence of a distinct fetal fibrinogen was first reported by Burstein and coworkers in 1954 (1). They compared some optical and mechanical properties of clots of newborn and adult fibrin and found that a clot of fibrin of newborns was more transparant and less compressible than a clot of fibrin of adults. The difference was relatively pronounced at pH 8.6 but hardly detectable at pH 6.9. Künzer (2) noted in 1961 that whereas the level of most clotting factors was low, the level of fibrinogen in the newborn was usually the same as in the adult, however Künzer also noted differences in properties. He postulated a functionally different fibrinogen in the newborn with an accelerated fibrinogen-fibrin transition. Witt (3-9) isolated fibrinogen from human umbilical cord blood.

She found a significantly retarded thrombin-catalyzed conversion of cord fibrinogen to fibrin at pH's around 7. The difference was even more pronounced at pH 8.5. Electronmicroscopic comparison of fetal fibrin clots with those from adults showed shorter and thinner fibers, which might be a corrolary of the old observation that the fibrin clots in newborn plasma are more transparant (8,9).

Galanakis (10-12) emphasized the importance of the solvent conditions, especially of the ionic strength in the testing systems. He found that relatively high ionic strengths prolong the thrombin clotting time of cord fibrinogen more than that of adult fibrinogen. In agreement with Guillin (13) he found a slower rate and a lower degree of aggregation of fetal fibrin monomers as compared with adult fibrin monomers at high ionic strength. Teger-Nilsson (14) found only a slightly lower aggregation rate of fetal fibrin monomers and no significant prolongation of the thrombin clotting time at a physiological ionic strength and pH. This was also reported by Krause (15). Teger-Nilsson found no differences between the two types of fibrinogen on polyacrylamide gelelectrophoresis and mapping of tryptic peptides of reduced and alkylated polypeptide chains.

Witt and coworkers (3-9) described extensively the molecular properties of purified cord fibrinogen. The overall aminoacid composition of newborn fibrinogen was virtually the same as that of adult fibrinogen. On the basis of data obtained by fingerprinting tryptic digests, she postulated that at least three peptides differ in newborn as compared with adult fibrinogen. Fetal fibrinogen has been reported to contain the same amount of hexose, but almost twice the amount of phosphorus of adult fibrinogen (5). This probably did not explain the functional differences because enzymatic dephosphorylation did not correct the functional properties of fetal fibrinogen. On DEAE cellulose column chromatography the major fibrinogen peak from newborn fibrinogen preparations eluted somewhat later than those obtained from adult material. The apparent K_m for thrombin was lower for fetal than for adult fibrinogen, suggesting a greater affinity of thrombin for fetal than for adult fibrinogen.

In 1979 Galanakis reported that partial removal of sialic acid shortened the prolonged thrombin clotting time of fetal fibrinogen. He found the sialic acid content of fetal fibrinogen to be higher than that of adult fibrinogen (12). This was also reported by Lane, who described a markedly different carbohydrate composition of fetal fibrinogen, particularly an increased sialic acid content (16). Galanakis reported in 1977 that a mixture of adult and fetal fibrinogen is found in the cord plasma of full-term infants. In premature infants there is relatively more fetal fibrinogen (11).

Mills and Karpatkin (17) challenged the existence of a distinct fetal fibrinogen. They concluded that the delay of clotting of cord fibrinogen is due to a greater content of preformed fibrin in these preparations and could not relate the heterogeneity of adult fibrinogen to the persistance of a fetal fibrinogen. Also other authors questioned the existence of a distinct fetal fibrinogen (18,19,20). Loly (21) could not find any difference between adult and fetal sheep-fibrinogen.

Altogether there is still a lot of controversy about the properties of the so called fetal fibrinogen and even its existence is doubted. Indeed the variations in experimental results can be explained by differences in

purification methods and solvent conditions in the testing systems. Also artefacts, introduced by limited proteolysis in vitro cannot be precluded. We therefore reexamined the problem under conditions, where special care was taken to prevent proteolysis in vitro.
We also measured the influence of adding increasing amounts of fibrinogen degradation product fragment X to see whether the reported differences between preparations of newborn and adult fibrinogen could be explained by the presence of degradation products in the former.

Materials and Methods

Blood samples were obtained from the umbilical cords of normal full-term infants by venapuncture, immediately after clamping the umbilical cord. The Apgar score of the infants was more than 7 after one minute, the pH of the umbilical vein blood greater than 7.20 and the birth weights were all in the normal range. There were no signs of fetal distress before parturition and no macroscopic alterations of the placenta and the umbilical cord were observed.
The mothers did not receive any medication, reported to possibly influence the haemostatic reaction in the child.
The first few ml's of cord blood after a clean venapuncture were discarded. The blood was then collected in an anticoagulant medium containing 1%(w/w) Na_2 EDTA, 0.9%(w/w) NaCl and 400 K.I.U. Trasylol per ml. 9 volumes of blood were mixed with 1 volume of the anticoagulant medium.
Immediately after blood sampling 1 % (v/v) di-isopropylphosphofluoridate (DFP) solution (0.1 M in dry isopropanol) was added to prevent proteolysis in vitro. The samples were centrifuged for 15 minutes at 1500 g in a swing-out rotor. The plasma was centrifuged at 20.000 g for twenty minutes to remove blood platelets and other particulate elements.
The plasma was then diluted to 1.5 times the original blood volume with a phosphate buffer, 0.15 M, pH 7.5, containing per liter 22.3 gr. K_2HPO_4, 3 gr. KH_2PO_4, 1.1 gr. Na_2EDTA, 0.1 gr. NaN_3 and 20.000 K.I.U. Trasylol. At each step 1 % (v/v) DFP solution was added.
Cord plasma's of five newborns were combined to give a pool of approximately 100 ml plasma and stored at -20 °C untill further use. The cord blood was obtained with the kind coöperation of the staff of the Midwivery School in Heerlen (Head: Dr. R. Oomers).
Adult blood was collected and plasma prepared from healthy donors as described above.

Purification of Fibrinogen:

Fetal and adult fibrinogen were purified from 100 ml cord plasma and adult plasma respectively, by the procedure to be described below. The plasma was applied to a Sepharose-lysine column (2.8x13 cm) to remove plasminogen. The eluate was brought to 25% saturation in $(NH_4)_2SO_4$ (13.9 gr./100 ml), stirred for one hour at 4 °C and centrifuged for twenty minutes at 3000 g. The

precipitate was dissolved in phosphate buffer to 1.5 times the original blood volume and again precipitated at 25% $(NH_4)_2SO_4$ saturation. This precipitate was dissolved in a minimum volume of phosphate buffer and gel filtered on a Sepharose 6B column (2x120cm) at 4 °C. The second peak (absorbance at 280 nm) contains virtually pure fibrinogen. The purification procedure has been described in detail elsewhere (Van Ruyven-Vermeer et al. 22).

SDS polyacrylamide gelelectrophoresis was performed according to standard procedure with and without β-mercaptoethanol (23).

Thrombin clotting time: Fibrinogen concentrations were adjusted to 1 mg/ml. Aliquots of 0.200 ml fibrinogen solutions were preincubated for 30 seconds at 37 °C. The reaction was started with 0.050 ml of a bovine thrombin solution (5 N.I.H.U/ml)(Roche, Basel) and the clotting time recorded with the use of a Kolle Hook.

The influence of the pH of the fibrinogen solutions on the thrombin clotting time was studied by varying the pH by dialyzing before the experiment against buffers containing 0.15 M NaCl and 0.01 M Tris. Ionic strengths were varied in the range of 0.05 to 0.30 M NaCl at a constant pH 7.4. The influence of calcium added to the thrombin solution was also studied. To estimate the K_m, the clotting times were obtained with fibrinogen solutions of different concentrations.

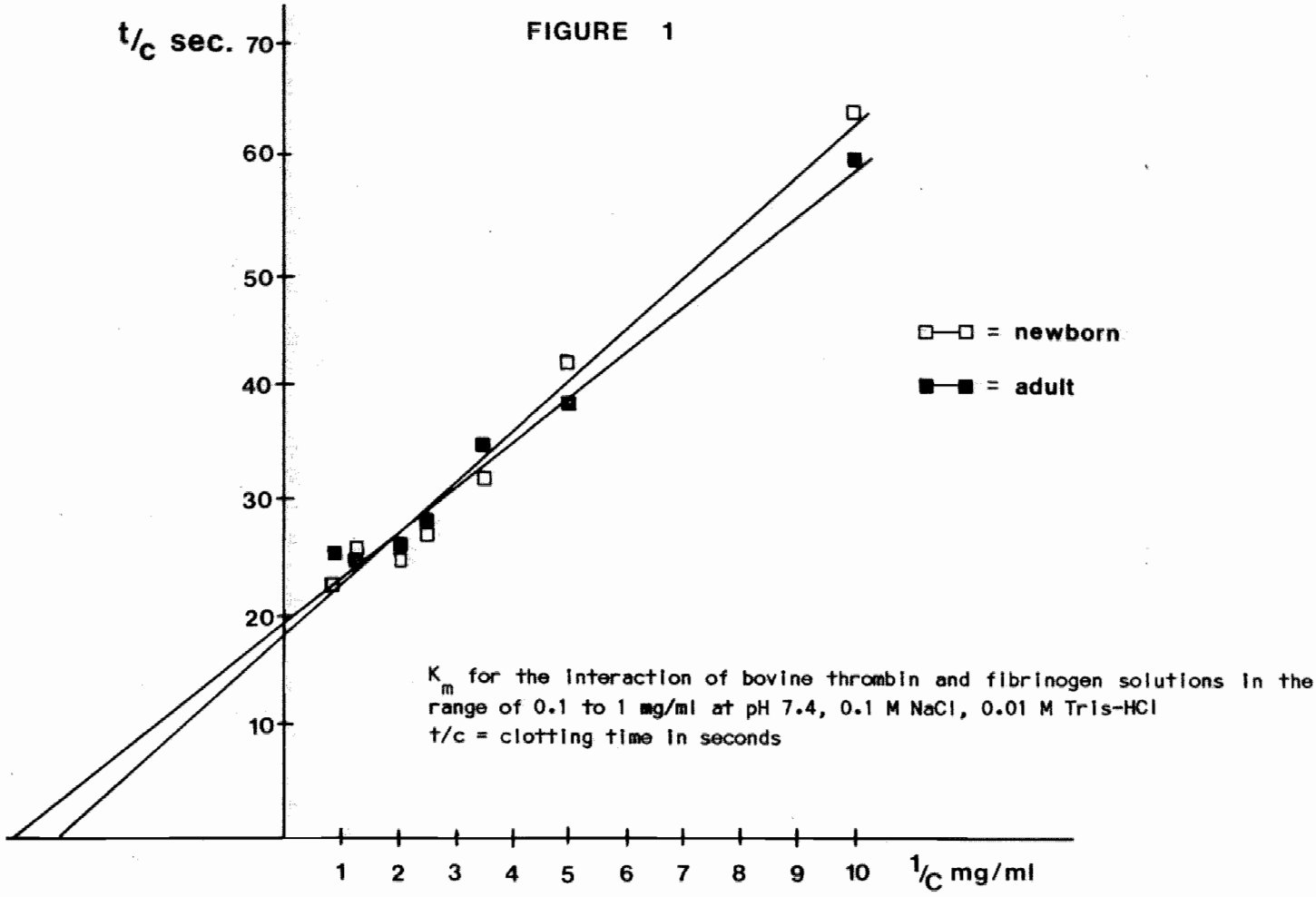
Fragment X was prepared by limited plasmin digestion of purified adult fibrinogen and purified according to W. Nieuwenhuizen and M. Gravesen (24). The effect of adding increasing amounts of fragment X on the thrombin clotting time of cord and adult fibrinogen was studied, especially under conditions with a high pH and high ionic strength.

Carbohydrate determination: the carbohydrate analyses were performed essentially as described by Kamerling and Clamp (25). They were carried out by Drs. J.P. Kamerling, G.J. Gerling and J.F.G. Vliegenthart, Laboratory of Organic Chemistry, University of Utrecht, Utrecht, The Netherlands. Before analysis samples of fibrinogen were run through a Biogel-P 60 column in 0.15 M NaCl, dialysed extensively against several changes of distilled water and freeze-dried.

Phosphorus determination: phosphorus determinations were carried out according to Böttcher (26). Before analysis samples of fetal and adult fibrinogen were dialyzed against several changes of twice-distilled water and freeze-dried. To check the possibility that the phosphorus in the fibrinogen originates from bound phospholipids, the fibrinogen preparations were extracted according to the following procedure: 20 mg portions of fetal and adult fibrinogen in 10 ml 0.15 M NaCl were dialyzed against distilled water and freeze-dried.

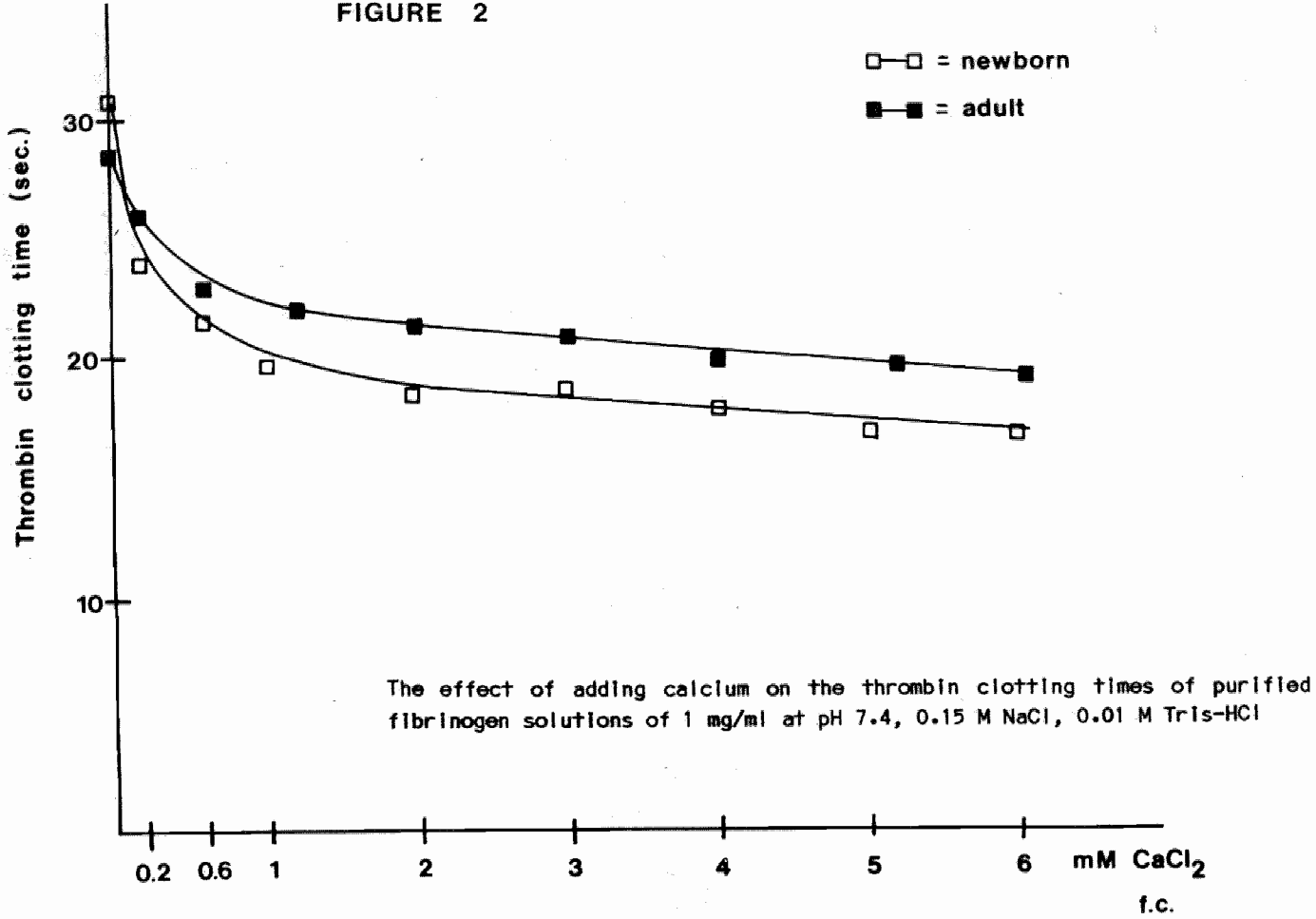
10 ml NaCl (0.15 M) was also dialyzed and freeze-dried, to serve as a control. To the residues 40 ml methanol: chloroform = 1:2 was added and the mixture heated for 15 minutes at 60 °C. After cooling to room temperature 9 ml 0.15 M NaCl was added to each tube and shaken well.

FIGURE 1



K_m for the interaction of bovine thrombin and fibrinogen solutions in the range of 0.1 to 1 mg/ml at pH 7.4, 0.1 M NaCl, 0.01 M Tris-HCl
t/c = clotting time in seconds

FIGURE 2



The effect of adding calcium on the thrombin clotting times of purified fibrinogen solutions of 1 mg/ml at pH 7.4, 0.15 M NaCl, 0.01 M Tris-HCl

After centrifugation to separate the phases, the upper layers were removed, the bottom layers were washed once with methanol: chloroform: water = 48:4:47 and centrifuged again. The remaining bottom layers were evaporated under a stream of nitrogen, taken up in 0.5 ml chloroform and the amount of phospholipids determined according to van Gent (27).

Results and Discussion

In contrast with some reports in the literature we found no significant different influences of the pH and/or the ionic strength on the thrombin clotting time of adult and cord fibrinogen (results not shown).
Only at high ionic strength (0.30 M NaCl) and high pH (9.0), the thrombin clotting time seems to be slightly prolonged with cord fibrinogen as compared with adult fibrinogen. However, it is difficult to decide how significant these differences are, since it was difficult to assess the clotting times accurately under these conditions.
The pH optimum for the thrombin-catalysed transition of both adult and cord fibrinogen to fibrin was 7.4.
The K_m values for the interaction of thrombin (bovine) with adult and cord fibrinogen were determined by measuring clotting times with varying fibrinogen concentrations at a fixed thrombin concentration. The results are shown in figure 1. In this figure use has been made of the fact, that the clotting time is proportional to 1/v (v = velocity of the overall fibrinogen conversion reaction in an experimental set up)(28). As can be concluded from this figure, no significant differences could be found between the K_m's of adult and cord fibrinogen. The values found were 0.70 and 0.62 μM respectively.
Electrophoresis on SDS polyacrylamide gels containing β-mercapto-ethanol showed no difference between adult and cord fibrinogen (not shown).

TABLE I

Carbohydrate composition of adult and cord fibrinogen

	nmol./mg		molar ratio	
	adult	cord	adult	cord
mannose	40.7	31.7	3.0	3.0
galactose	25.5	19.5	1.9	1.8
glucose	10.7	9.9	0.8	0.9
N-acetyl glucosamine	32.3	24.7	2.4	2.3
sialic acid	16.5	14.5	1.2	1.4

The carbohydrate compositions of cord fibrinogen and adult fibrinogen are essentially the same (table 1) and the results are in accordance with the

data of Townsend (29) for adult fibrinogen.
Differences in the sialic acid content, reported in the literature (12,16) were not confirmed.

We could confirm that the phosphorus content is 3 to 4 times higher in cord fibrinogen than in adult fibrinogen (5). The results are shown in table 2. It could be excluded that this was due to contamination with phospholipids, because after lipid extraction virtually no phosphorus was found in the extracts.

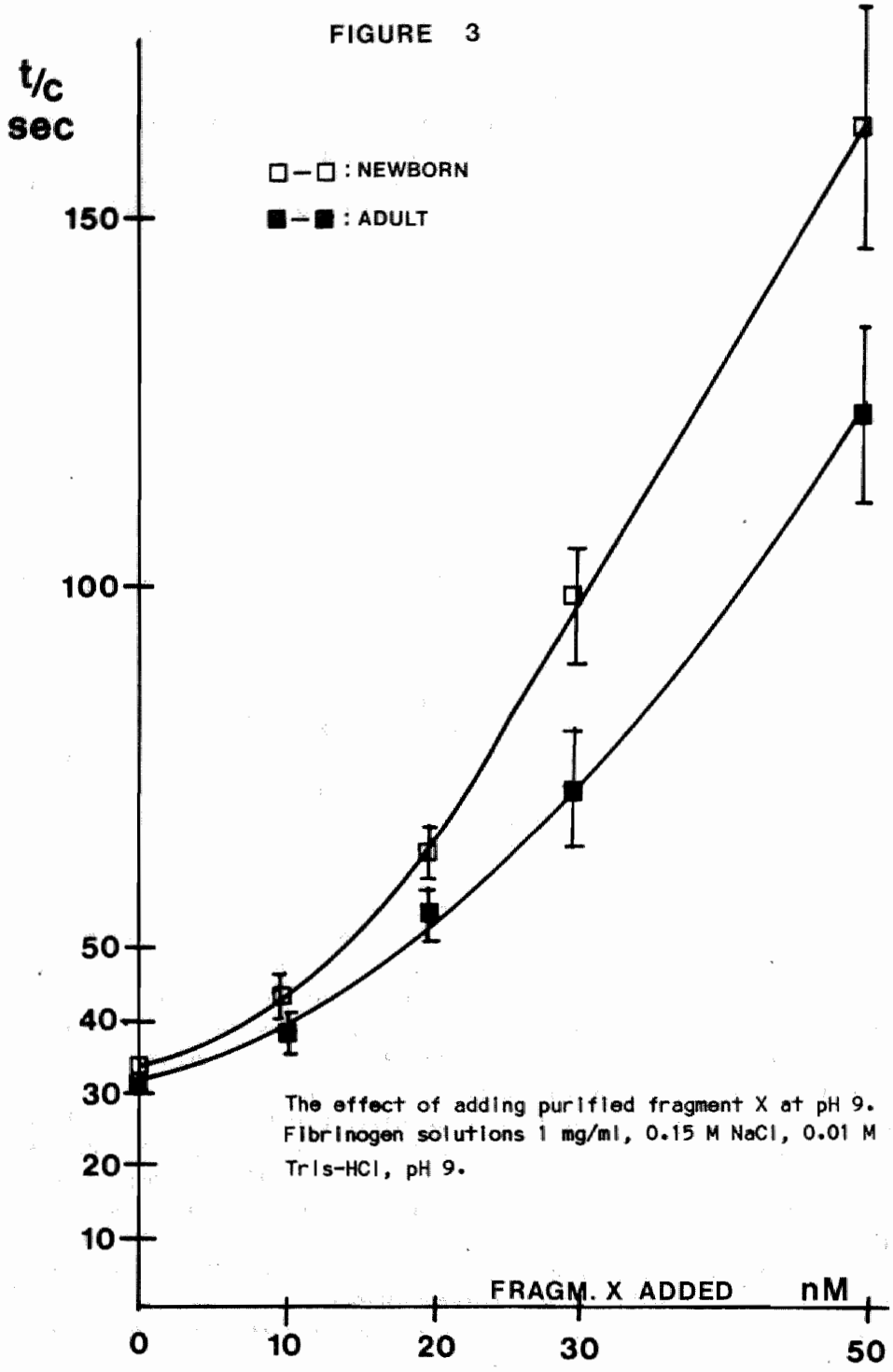
TABLE II

Phosphorus content of adult and cord fibrinogen (molar ratio)

cord fibrinogen:	P = 1 : (10.9 ± 0.2).
adult fibrinogen:	P = 1 : (2.9 ± 0.4).

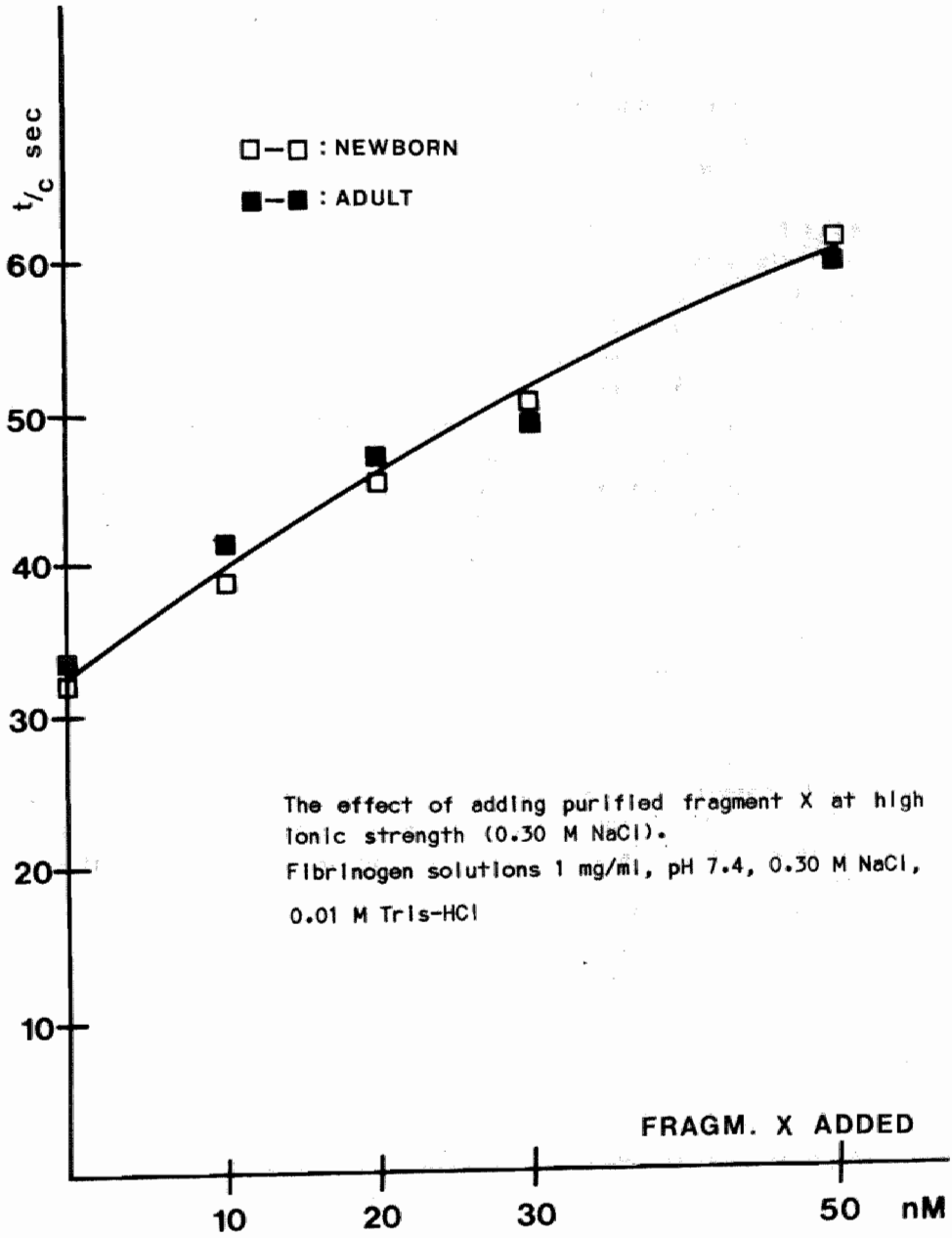
We also studied the influence of calcium on the thrombin-catalyzed transition of fibrinogen to fibrin. The results are shown in figure 2.
From this it can be seen that the procoagulant effect of adding calcium to the thrombin solution is more pronounced in the preparations of cord-fibrinogen than in those of adult fibrinogen.
Some of the properties, ascribed to a distinct fetal fibrinogen species, might be explained by the occurence of contaminants, especially fibrinogen breakdown products, in some cord fibrinogen preparations. These might have been formed in vivo and/or in vitro. We tried to minimize fibrinogen proteolysis in vitro in our purification procedure.
No fibrinogen degradation products were observed in our purified cord and adult fibrinogen preparations. This indicates that such degradation products do not occur in vivo in detectable amounts or that they have been lost during the purification of the fibrinogen. The latter possibility would lead to a correction of the apparent abnormalities observed with cord plasma as a result of the purification.
The occurence of degradation products in newborn blood in vivo is likely, since it is known that in the sick newborn a marked degree of diffuse intravascular coagulation is quite common, probably related to hypotension, hypothermia, hypoxia, acidosis, poor tissue perfusion, hepatic immaturity and a poorly developed reticuloendothelial system, which may prevent adequate clearing of activated coagulation factors and therefore enhance the sequala of diffuse intravascular coagulation.
The enhanced fibrinolytic capacity of cord blood of normal newborns is well

FIGURE 3



The effect of adding purified fragment X at pH 9. Fibrinogen solutions 1 mg/ml, 0.15 M NaCl, 0.01 M Tris-HCl, pH 9.

FIGURE 4



The effect of adding purified fragment X at high ionic strength (0.30 M NaCl).
Fibrinogen solutions 1 mg/ml, pH 7.4, 0.30 M NaCl, 0.01 M Tris-HCl

documented in the literature (30) and may be the cause of some proteolysis of fibrinogen.
If demonstrated, the presence of degradation products in cord plasma could explain some of the properties, ascribed to fetal fibrinogen. It is known, that fragment X, for instance is a potent inhibitor of the action of thrombin on fibrinogen and polymerization of fibrin monomers (24). In nearly all newborn cord plasma's the thrombin clottting time is prolonged. According to our data, this prolongation cannot be attributed to different clotting properties of purified cord fibrinogen, but might well be explained by the presence of for instance fragment X. As at the time of this study no specific and sensitive methods were available to demonstrate the presence of low levels of some of the high molecular weight breakdown products directly in plasma, we studied the influence of increasing amounts of added fibrinogen breakdown product fragment X to the solutions of purified fibrinogens, especially under conditions with a high pH and high ionic strength.
As can be seen in figure 3, the thrombin clotting time of cord fibrinogen appeared to react stronger to the addition of fragment X than adult fibrinogen at pH 9. No differences were recorded between the two types of fibrinogen at high ionic strength, when increasing amounts of fragment X were added to the fibrinogen solutions. This is shown in figure 4.
These results suggest that some of the clotting properties, ascribed in the literature to fetal fibrinogen may be due to a contamination with degradation products such as fragment X.
Witt postulated (9) that the synthesis of fetal fibrinogen lasts only for 7-8 days after birth. We suggest, that after that period the fibrino(geno)-lysis has returned to normal levels and that degradation products such as fragment X have been cleared from the newborn blood.
Our results clearly confirm the higher phosphorus content in purified cord fibrinogen than in adult fibrinogen. Little or nothing is known about the role of phosphorus in the fibrinogen molecule.
Blombäck (31) showed that the phosphorus in adult fibrinogen is covalently bound in the form of O-phosphoserine and partly found in fibrinopeptide A. According to the data of Witt enzymatic dephosphorylation had no effect on the functional properties (5).
The higher phosphorus content may play a role in the more pronounced effect of calcium added to the thrombin solution, on the thrombin clotting time in purified cord fibrinogen as compared with adult fibrinogen. At present experiments are carried out to find possible correlations between levels of plasminogen activators and prolonged thrombin clotting times of cord plasma

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CHAPTER 8

A NEW CASE OF A CONGENITAL COMBINED HYPO- DYSFIBRINOGENAEMIA

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Summary

In this paper we present the preliminary results of a study of a new case of a combined hypo- dysfibrinogenaemia. Because of an umbilical stump haemorrhage two days after birth, a screening profile of the haemostatic mechanism was performed on a female child of dutch origin. This showed a very low amount of thrombin clottable fibrinogen, measured according to the method of Clauss (20). No other abnormalities were found. Fibrin(ogen) degradation products could not be detected in plasma, using specific monoclonal antibodies. The reptilase clotting time was also prolonged, compared with normal values found at her age. However, reptilase induced fibrin clot formation appeared to be less abnormal than thrombin induced fibrin clot formation.
In mixtures of normal plasma and plasma of the propositus no inhibitory effect on the thrombin clotting time of normal plasma was found. Immunological studies showed that the amount of fibrinogen related antigen was at least 2.5 fold higher than the amount of thrombin clottable fibrinogen. The discrepancy was much higher in "heat defibrinated" samples, suggesting that the heating procedure leads to a major change in the antigenic sites of the abnormal fibrinogen molecule. Crossed immunoelectrophoresis showed also an abnormal form and a slightly different position of the precipitation arc of the plasma of the propositus in the absence and presence of calcium.
Until now (age 3) our patient has remained asymptomatic and her development was normal. This case underlines the hypothesis of Ménaché (1) that many of the hypofibrinogenaemias are in fact partly dysfibrinogenaemias.
It is remarkable that in our case the family history appeared to be negative. This is very unusual, as most of the hypo- and dysfibrinogenemias follow a dominant inheritance pattern. For practical reasons we decided to postpone a definitive purification and characterization of the fibrinogen of the propositus and her family.

Introduction

Congenital fibrinogen abnormalities cover a great variety of rare disorders, including afibrinogenaemia, hypofibrinogenaemia and dysfibrinogenaemia. Afibrinogenaemia and hypofibrinogenaemia are characterized by a lack or important decrease of circulating functionally normal fibrinogen, whereas dysfibrinogenaemia is characterized by the presence of qualitative abnormal and functionally defective fibrinogen molecules. Dysfibrinogenaemia is usually classified according to the functional defect present, for instance abnormalities in the release of the fibrinopeptides, abnormal polymerization of fibrin monomers, abnormal crosslinking of fibrin by factor XIIIa or abnormal sensitivity to plasmin. Recently it has been postulated that hypofibrinogenaemia is not necessarily a partial deficiency

of the normal fibrinogen molecule only, but that in a number of cases it should be classified as a dysfibrinogenaemia, or as a combined hypo-dysfibrinogenaemia (Ménaché 1983) (1). Since the first well documented case of a dysfibrinogenaemia was published in 1963 (2), approximately 100 families have been described (1,3,4). With the exception of fibrinogen Oslo 1 (5), in which conversion is accelerated, fibrinogen to fibrin conversion is delayed in abnormal fibrinogens, the common finding being a prolonged thrombin clotting time (1). The molecular defect has been characterized for several among them. It often involves a single amino acid substitution in the Aα chain (6-11). All these fibrinogens exhibit delayed release of fibrinopeptide A. No single amino acid substitution has been reported so far in the Bβ or the γ chains. Other structural defects have also been described, for instance a segmental deletion in the carboxy terminal end of the Aα chain (12), defective γ chains and elongation of the γ chain (13-15). In these abnormal fibrinogens, the fibrin monomer aggregation is delayed, whereas the release of the fibrinopeptides is normal. Also other abnormalities have been described in these variants of human fibrinogen, such as an inhibitory effect on the thrombin clotting time of normal plasma and a hypercatabolism of fibrinogen which may or may not be compensated by enhanced fibrinogen synthesis (28). Afibrinogenaemia has been described for the first time in 1920 (16). The total number of families reported since that time is around 70 (1). Hypofibrinogenaemia, first recognized in 1935 (15) has a much lower incidence (1). Congenital abnormalities of fibrinogen occur in the same frequency in both sexes. The mode of inheritance is autosomal dominant for the group of the afibrinogenaemias. In the case of hypofibrinogenaemia the mode of inheritance is less well defined, probably as a result of the heterogeneity of this disorder (1). In some families, absence of consanguinity between the parents, haemorrhagic symptoms in one of the parents or low fibrinogen levels in only one of the parents, favor dominant transmission. In other families a recessive transmission has been postulated based on consanguinity between parents, low fibrinogen levels and lack of clinical symptoms in both parents. In dysfibrinogenaemia the mode of inheritance is autosomal dominant, except for fibrinogen Parma and fibrinogen Valencia (18,19). In this report we present the preliminary results of a study of the fibrinogen of a child, that at birth showed a remarkable low level of thrombin clottable fibrinogen, measured according to ref. 20.

Case history

The propositus was born in January 1983 as the second female child of a 28 year old mother and a 29 year old father. Pregnancy and parturition were normal. During pregnancy the mother used an oral iron therapy as suppletion. No reasons could be found to assume intoxications known to influcence the haemostatic mechanism in the offspring.

The child was born à terme via vaginal delivery, birth weight 3400 gram, length 50 cm, the Apgarscore was 9 after 1 minute and 10 after 5 minutes. A routine physical examination showed no abnormalities. Two days after birth the umbilical stump started bleeding, without evidence for trauma, infection or any other local pathological process. There were no other bleeding sites and a physical examination showed no other abnormalities at that time. The child was fed with breastmilk and had received no medication or vitamin suppletion. The bleeding was treated locally by suturing and local pressure. Because of the umbilical stump haemorrhage a screening profile of the haemostatic mechanism was performed in the routine laboratory of the hospital were delivery had taken place. The results of this screening are summarized in table I.

TABLE I

	Patient	Normal values two days after birth
haematocrit	54%	60% ± 6.4
bleeding time	2' 15"	1-4'
thrombocytes	317 x 10^9/l	150-400 x 10^9/l
thrombotest	25%	20-60%
aPTT	65"	40-70"
F VIII	76%	80-120%
fibrinogen (Clauss)	0.2 g/l	1.4-3.2 g/l

During 8 days the bleeding site remained oozing. Until now no recurrent bleeding problems have occurred and the development of the child was entirely normal. Impaired wound healing was not reported. It was remarkable that the family history appeared to be negative, although on the father's side a newborn child died a few days after birth, without a known cause. There was no known consanguinity within the family. We reinvestigated the patient at the age of 3 months and one year as well as the only sibling (female, 2 years) and both parents.

Materials and Methods

Blood was obtained by a clean venapuncture. After discarding the first few ml's, blood was collected (9 volumes) in a plastic tube containing anti-coagulant (1 volume, 3.2% disodium citrate dihydrate) for the coagulation studies and in a mixture of diluted human thromboplastin, Trasylol and EACA for the determination of fibrin(ogen) degradation products. The blood was centrifuged 2000xg for 15' and platelet free plasma was obtained by

centrifugation for 30' at 20.000 g at 4 °C. The plasma's were stored at -70 °C until further use. Prothrombin time (PT) was measured using a human brain thromboplastin, prepared in our laboratory. The partial thromboplastin time (PTT) was measured with a aPTT reagent purchased from Dade, Salm en Kipp, Breukelen, Holland. The tests were performed as described previously (21,22). Thrombotest® and Normotest® were performed according to the manufacturers prescription (Nyegaard, Oslo, Norway) as described by Owren (23). Bovine thrombin was obtained from Hoffman La Roche, Basle, Switzerland. The thrombin clotting time was measured by adding 0.05 ml of a thrombin solution (5-10 N.I.H.U/ml) to 0.2 ml plasma after 30" preincubation of the plasma at 37 °C. The clotting time was recorded manually with the Kolle Hook. The fibrinogenlevel was determined according to the method of Clauss (20) (biological activity) and by rocketimmunoelectrophoresis, using human antifibrinogen antibodies purchased from Behringwerke, Marburg, FRG. The rocketimmunoelectrophoresis was performed according to Laurell (24). We also estimated the fibrinogen concentration on basis of the clotting times obtained with the snake venom Reptilase® (Boehringer Mannheim, Amsterdam, Holland). Reptilase® reagent (0.1 ml, prewarmed) was added to 0.3 ml plasma after preincubation at 37 °C for 2 minutes. The clotting time was manually recorded with the Kolle Hook. Heat defibrinated samples were prepared according to Hensen and Loeliger (25) and also tested for the amount of fibrinogen related antigen (FRA) by rocketimmunoelectrophoresis.

Fibrin(ogen) degradation products were determined in a conventional assay, using the Thrombo-Wellco test kit (Wellcome Res Lab, Beckenham, England) (sensivity limit 10 µg/ml) as well as with monoclonal antibodies against neoantigens which are formed during proteolytic breakdown of fibrinogen and fibrin as described by Mirshahi (26).

These assays were kindly performed for us by Dr. Massoud Mirshahi (Hôtel Dieu, Paris, France). Factor XIII levels were determined in a semi-quantitative way, using a commercial test (Boehringer Ingelheim, FRG) according to the manufacturers prescription. Crossed immunoelectrophoresis against human antifibrinogen antibodies (antibody concentration 0.5 vol%) was performed according to Laurell (27) in the absence and presence of calcium lactate (2 mM).

Results

In table 2 the data of the coagulation studies of the propositus and the family are summarized and compared to normal pool plasma and normal values for the age of the propositus. Fibrin(ogen) degradation products could not be demonstrated in any person of the family neither in the conventional assay, using the Thrombo-Wellco test kit, (Wellcome Res. Lab, Beckenham, England), nor using a method based on recognition of neoantigens, that are

TABLE II

	Ref. adult normal plasma	father	mother	sibling	proband	normal values for the age
PT (Quick) (sec)	14.9	15	15.1	16.1	42.6	16-22
aPTT (sec)	34.0	33.8	33.5	34.9	48.4	32-50
reptilase time	17.5	18.0	16.4	21.0	45.2	24-36
Thrombin time 5 U (sec)	19	20.5	21.8	21.4	53.4	20-32
Thrombin time 10 U (sec)	14	15.7	15.0	16.5	21.5	18-24
FDP's	negative	negative	negative	negative	negative	negative
Normotest^{R} (%)	100%	100%	110%	90%	70%	~50%
Thrombotest^{R} (%)	100%	100%	108%	80%	66%	~50%
Fibrinogen (gr/l)	3,4 g/l	2,0 g/l	2,5 g/l	2,1 g/l	0,2 g/l	1,6-3,5 g/l
	(biological activity according to Clauss)					

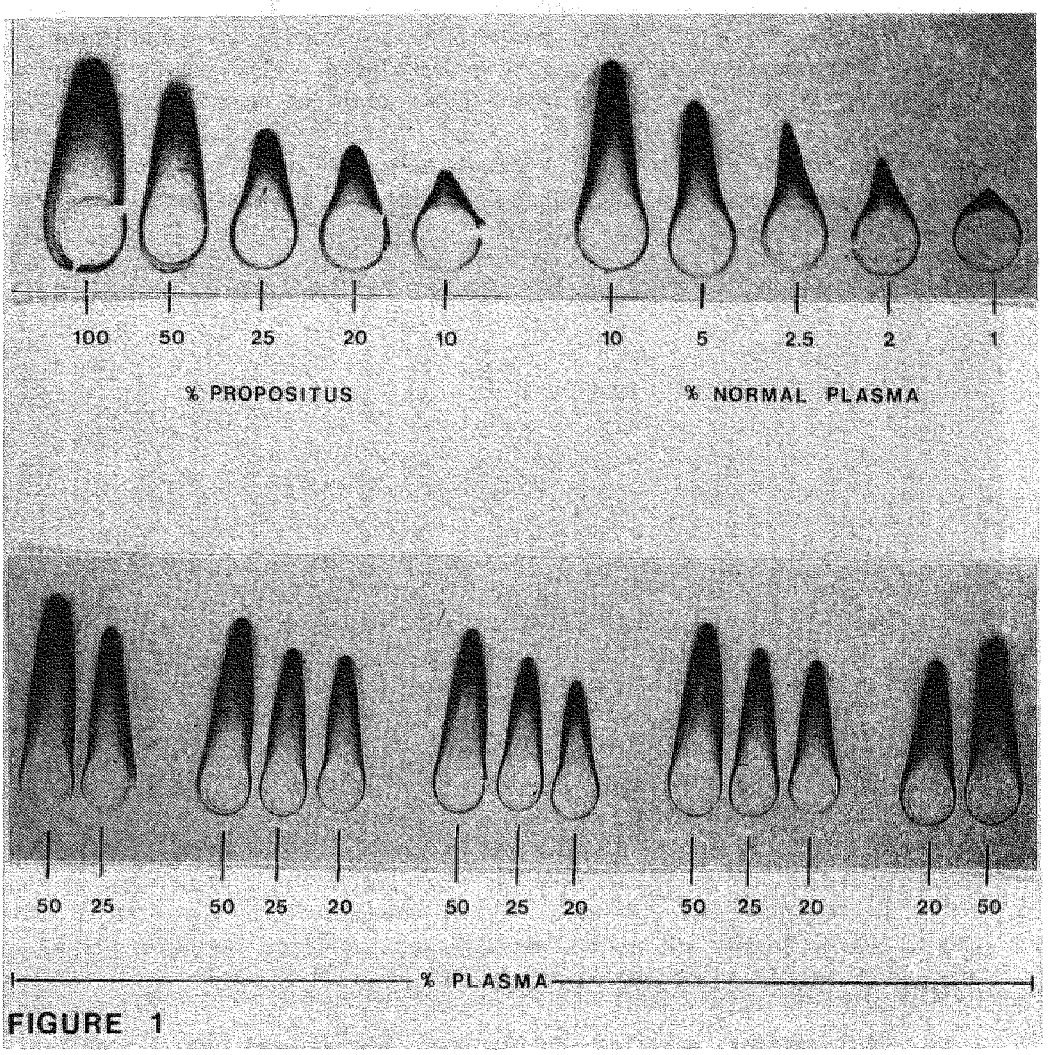


FIGURE 1

Rocketimmunoelectrophoresis of the propositus (upper side) and her family and normal plasma against antifibrinogen antibody.
From left to right: normal plasma, father, mother, sibling, normal plasma

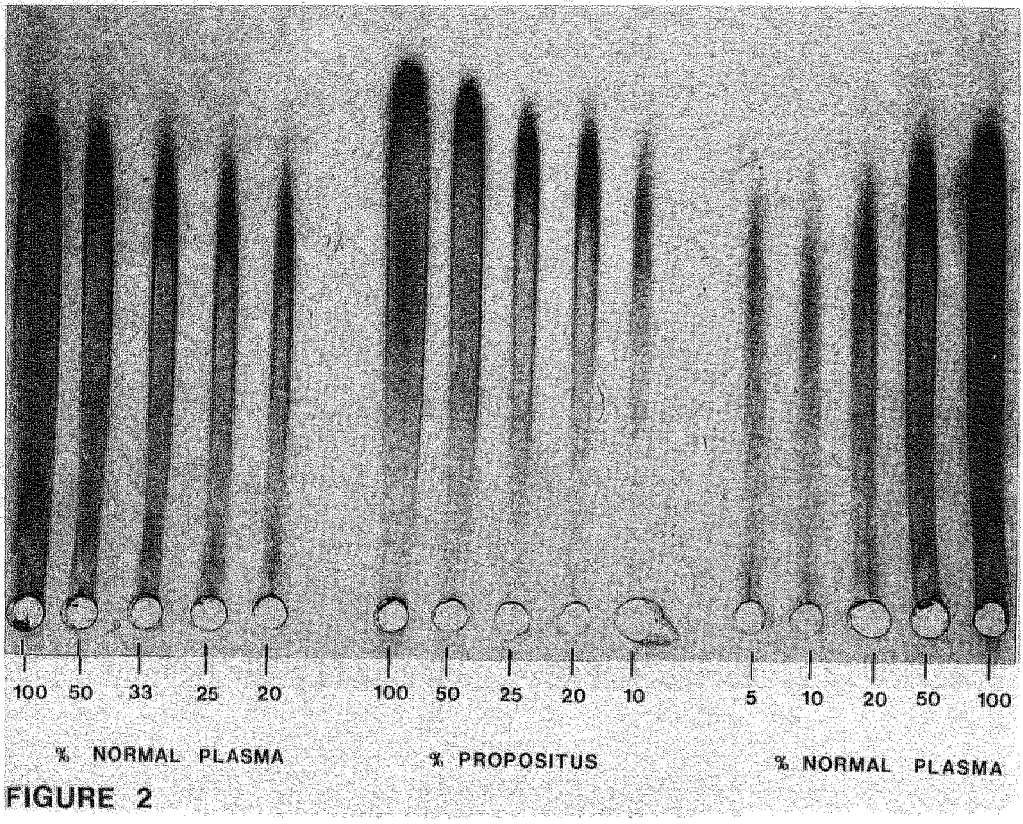


FIGURE 2

Rocketimmunoelectrophoresis of heat defibrinated plasmas

exposed as the result of proteolytic breakdown of fibrinogen and/or fibrin, by monoclonal antibodies (26). This method detects F.D.P. at levels of 0.25 µg/ml and higher. Also in this assay no proteolytic breakdown products of fibrin or fibrinogen could be demonstrated in the plasma of the propositus and her family.

Thrombin clotting times were also measured of mixtures of normal plasma and the patients plasma and the plasma's of the other members of the family. These data did not provide evidence for a circulating anticoagulant (results not shown). In a number of cases of abnormal fibrinogens, an inhibitory effect on the thrombin clotting time of normal plasma is found (28).

In figure 1 the rocketimmunoelectrophoresis is shown of dilutions of normal plasma, the propositus and her family, using human antifibrinogen antibody in a concentration of 0.5 vol%. The reading was difficult, because in the circumstances used for the electrophoresis, diffusion of fibrinogen related antigen (FRA) was also seen besides and under the starting point. The amount of fibrinogen related antigen (FRA) was at least 0.5 g/l. The ratio of FRA: fibrinogen level according to Clauss was therefore 2.5 or higher. In the plasmas of the other members of the family no significant discrepancy was noted between the FRA level and the fibrinogen level determined according to the method of Clauss (results not shown).

In figure 2 the rocketimmunoelectrophoresis of heat defibrinated plasmas shows that the FRA, estimated according to this method, appeared to be much higher than was expected from the results obtained with rocket immunoelectrophoresis of the non-defibrinated plasmas. Moreover, we observed a qualitative difference in the precipitation pattern of the propositus, compared to the plasmas of the family members and normal plasma.

In figure 3 we show crossed immunoelectrophoresis patterns of the plasma of the propositus and the family in the absence (panel A) and presence (panel B) of calcium. In the plasma of the propositus an abnormal form and slightly different position of the precipitation arc was observed. In the patient, the factor XIII level was at least 25%, which is low normal for her age (29).

In figure 4, the results are given of the reptilase clotting times, as a function of dilutions of normal plasma. Based on the fibrinogenconcentration, determined according to the method of Clauss, a reptilase clotting time of around 80 sec. would have been expected. We found, however, a shorter reptilase clotting time in our patient and estimated the fibrinogenconcentration, using the assay, to be around 0.65 gr/l. Reptilase induced fibrin clot formation in the propositus therefore appeared to be less abnormal, than thrombin induced fibrin clot formation.

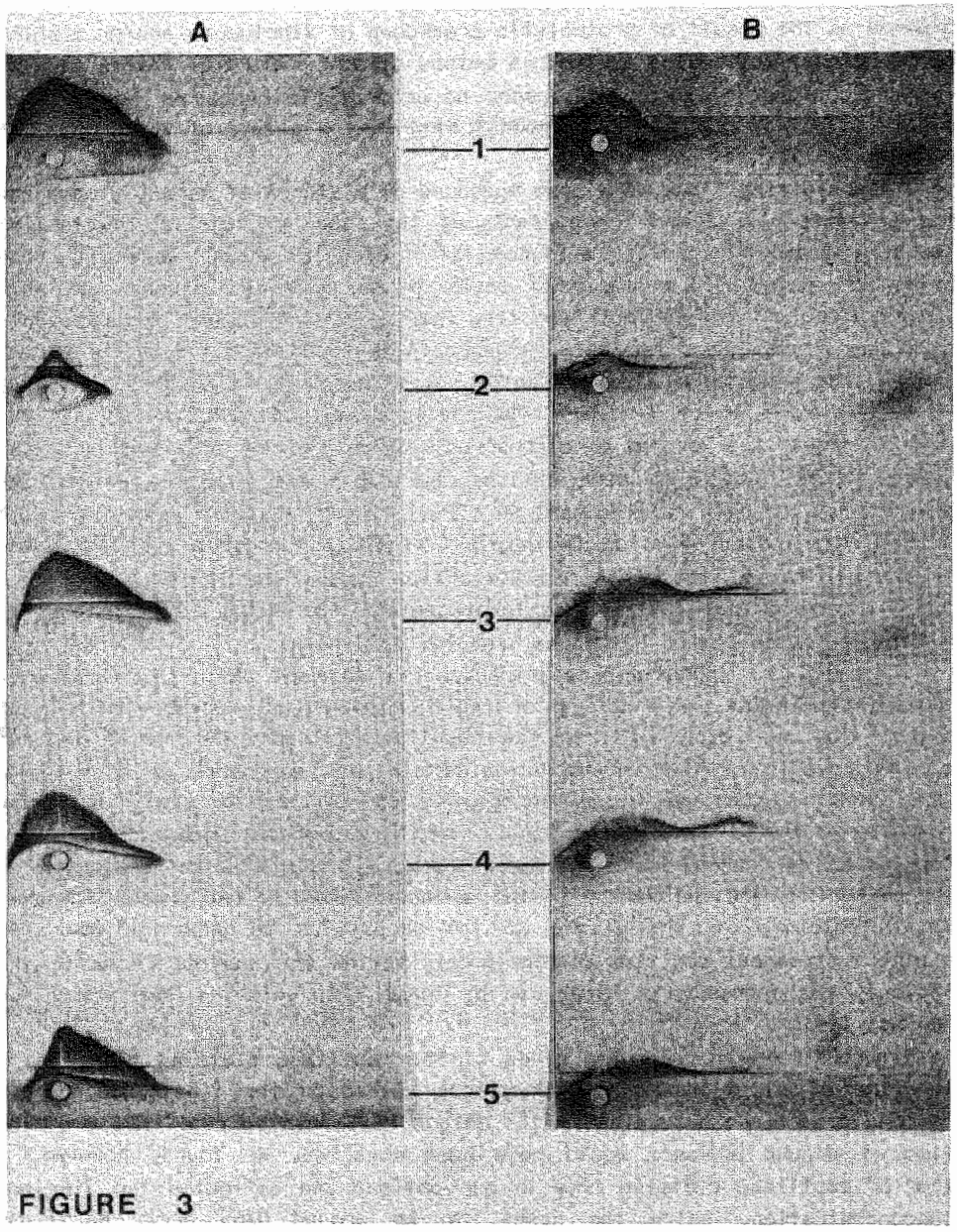
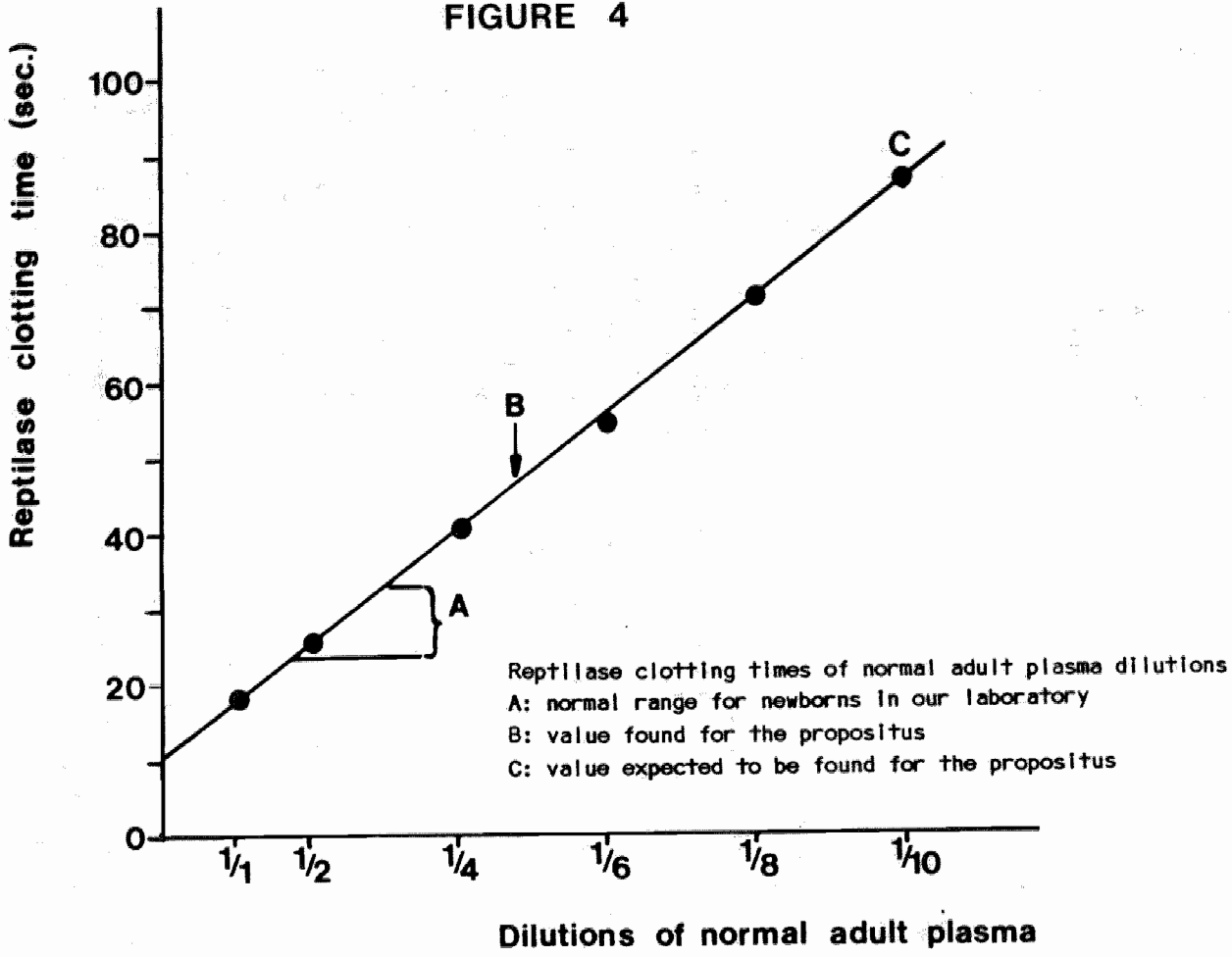


FIGURE 3

Crossed immunoelectrophoresis patterns against antifibrinogen in the absence (panel A) and presence (panel B) of calcium lactate (2 mM).

1. normal plasma
2. propositus
3. sibling
4. father
5. mother

FIGURE 4



Reptilase clotting times of normal adult plasma dilutions
A: normal range for newborns in our laboratory
B: value found for the propositus
C: value expected to be found for the propositus

Discussion

The case described must be considered to represent a combined hypo-dysfibrinogenaemia. The amount of fibrinogen related antigen (FRA) is distinctly higher than the amount of clottable fibrinogen. The discrepancy was especially clear in the "heat defibrinated" samples, suggesting that the heating procedure leads to a major change in the antigenic sites and a better recognition by the antibody. In our immunological studies we found also some evidence for qualitative abnormalities of the fibrinogenmolecule of the propositus. In the rocketimmunoelectrophoresis diffusion of FRA was observed under and besides the starting point. In the rocketimmunoelectrophoresis of the defibrinated plasma of the propositus the precipitation pattern differed from normal plasma. We also found an abnormal precipitation pattern and a slightly different position in the crossed immunoelectrophoresis of non-defibrinated and defibrinated plasma of the propositus.

Considering the obvious advantage of this procedure, it is surprising that only a small number of fibrinogen variants have been investigated by two dimensional electrophoresis (28). Another interesting observation was the fact that the reptilase induced fibrin clot formation of plasma of the propositus was only slightly retarded, when compared to the thrombin induced fibrin clot formation. Fibrin(ogen) degradation products could not be demonstrated, using the most sensitive methods available at the moment. The prolongation of the thrombin and reptilase clotting time in the plasma of the propositus could therefore not be attributed to the presence of fibrin(ogen) degradation products. We are aware of the fact, that our data are preliminary. For practical reasons (the need of 100 ml plasma) we decided to postpone a definitive purification and characterization of the fibrinogen, the more as there are no practical consequences for our patient involved. The family history in our case appeared to be negative. This is very unusual, as most of the hypo- and dysfibrinogenemias follow a dominant inheritance pattern. However, in a few families a recessive pattern has been postulated.

According to Ménaché (1) only a few cases of a pure hypofibrinogenaemia have been thoroughly investigated using extensive clotting and immunological methods. She has postulated, that many cases of hypofibrinogenaemia are in fact a combined hypo- dysfibrinogenaemia. A nice example of her hypothesis is the family, described by Hasselback in 1963, as being hypofibrinogenaemic, which appeared to be a dysfibrinogenaemia (Fibrinogen Vancouver, Thomas 1968) (31).

Patients with an afibrinogenaemia have a bleeding diathesis, especially in the neonatal period. The most frequent bleeding site is the umbilical stump. This has also been described in patients with a severe factor XIII deficiency. In our patient the factor XIII level was estimated to be 25% or more.

The clinical picture of dysfibrinogenaemia is less uniform than that of congenital afibrinogenaemia. 60% is asymptomatic and is found by chance, when a routine haemostasis screening is performed. Bleeding problems have been described, but have shown to be severe only for the fibrinogens Detroit and Bethesda III. A predisposition for thrombosis and for impaired would healing have also been described. Also the patients with hypofibrinogenaemia appear to be heterogeneous with regard to the clinical picture, as well as bleeding as thrombosis occur (1).

According to Ménaché (1) 20% of the cases of patients with hypofibrinogenaemia die of severe bleeding. In four patients multiple venous thrombotic problems with pulmonary embolism have been found, 2 of the 4 patients died of massive pulmonary embolism. It has been postulated that this thrombotic tendency is caused by an altered plasmin induced proteolysis on an abnormal fibrin surface. Until now our patient is asymptomatic and her development was normal.

We have presented this case, to underline the hypothesis of Ménaché that many of the hypofibrinogenaemias are in fact partly dysfibrinogenaemias.

Before claiming a new hypo- dysfibrinogenaemia it must be excluded that the molecular defect is the same as in other fibrinogen variants. Theoretically, it could be the same as described for fibrinogen Parma (18), fibrinogen Philadelphia I (32), fibrinogen Valencia (19), fibrinogen Giessen II (33) and fibrinogen San Juan (32). The hypo- dysfibrinogenaemia described by Owen (fibrinogen San Juan) was associated with van Willebrand disease (34). In our patient the bleeding time and factor VIIIc activity were normal at birth. Bleeding was often encountered in patients with these abnormal fibrinogen molecules (28).

Our preliminary results do not allow us at this moment to conclude that our case can be distinguished from the above mentioned abnormal fibrinogens. Unfortunately, the data in the literature are also often incomplete, making a comparison difficult or even impossible. We have planned in the near future a definitive purification and characterization of the fibrinogen of the propositus and her family.

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SUMMARY

Injury to the vascular endothelium is normally followed by haemostasis, i.e. the formation of a so called haemostatic plug. This process requires blood vessel support, adequate numbers and functioning of circulating blood platelets and sufficient quantities of a number of plasma proteins. This complex interaction is counterbalanced by a number of humoral and cellular protective mechanisms, in order to prevent an excessive reaction which would lead to an impaired blood flow, i.e., the development of thrombosis. Several key aspects of the haemostatic mechanism in newborn infants are different when compared with adult standards. Quantitative as well as qualitative differences have been described. Moreover, haemostatic problems, especially localized or generalized haemorrhages, are often seen in ill newborn infants. In spite of the rapid progress that has been made in the last 10 years in understanding the role and function of the various components of the haemostatic mechanism in adults, our knowledge of the haemostatic mechanism in newborns is still limited. In this thesis, several aspects of the haemostatic mechanism in newborns are discussed.

Chapter 1 summarizes briefly our up to date view of the haemostatic mechanism in adults. The role of the key enzyme thrombin in the haemostatic mechanism is especially emphasized. Next, the physiology of the newborn haemostatic system is reviewed. We have attempted to provide the most current and pertinent data available rather than exhaustively to compile the existing literature. In this chapter, we also discuss which mechanisms can be held responsible for the observation that the thrombin clotting time in newborns is prolonged. In our study, we found that in a small number of the cases (½-1%?) the inhibition of thrombin in umbilical cord plasma is caused by the presence of a heparin-like substance. We have partially purified this heparin-like substance and described several of its properties. The effect of the heparin-like substance appeared to be dependent on the presence of antithrombin III. The inhibitory effect was abolished upon incubation with heparinase, however not upon incubation with trypsin. In chromogenic substrate assays, the heparin-like inhibitor appeared to have a remarkable anti IIa (thrombin) effect and hardly any anti Xa effect. Until now, circulating heparin-like substances have only been described in several haematological malignancies. There were no obvious differences between the newborns with heparin-like activity in their umbilical cord plasma and those who did not.
All newborns in our study were healthy, full term infants born after an uneventful pregnancy and vaginal delivery.

Chapter 2 summarizes the literature on the vitamin K status of newborns. In the introduction, we discuss the mode of action of vitamin K. Next, which

mechanisms can be held responsible for a possible vitamin K deficiency in newborns. The methods available at the moment to diagnose a vitamin K deficiency are briefly discussed. Recently, the discussion in the literature on the vitamin K status of newborns has regained much attention. Especially in the American literature, it is propagated that all newborns should receive vitamin K at birth prophylactically. Based on the data available in the literature and some own observations, we conclude, that the administration of vitamin K at birth to healthy, full term infants is only indicated, when the mother is vitamin K deficient, for instance resulting from fat absorption disturbances, lengthy use of antibiotics and malnutrition. The administration of vitamin K is also indicated, if there are drugs circulating in the newborn that have an anti-vitamin K effect, such as oral anticoagulant drugs (coumarin derivates) or anticonvulsives. In the postnatal period, it may be considered to supply additional vitamin K orally to breast fed infants. There is an increasing amount of evidence in the literature, suggesting that breast fed infants are more at risk for the development of a vitamin K deficiency after birth, especially when the vitamin K pool of the mother is marginal. In The Netherlands this situation is probably exceptional.

In Chapter 3 we describe, that the thrombotest, a frequently used screening assay in clinical practice, does not give reliable information on the amount of the vitamin K-dependent coagulation factors in newborns. For instance, the correlation coefficient between the thrombotest clotting times and the prothrombin coagulation activity (IIc) levels is only -0.46. A modification and extension of the use of the thrombotest, is the so called thrombotest dilution curve. This curve indicates the relation between the clotting times obtained with the thrombotest reagent of several dilutions of plasma. In curves, obtained with dilutions of umbilical cord plasma, an inhibitor is found in 67% of the cases. This inhibitory effect is probably caused by the presence of breakdown products of fibrinogen and fibrin. These products are undetectable in umbilical cord blood when conventional methods are used. Recently new, much more sensitive methods, based on the use of monoclonal antibodies, have been developed, which enable us to determine these products directly in plasma. In all samples that showed no inhibition in thrombotest dilution curves, this test appeared to be negative, whereas it was positive in inhibitory samples. The most striking was the finding that in these inhibitory samples also fibrinogen breakdown products were present. We could imitate the inhibitory effect by adding small amounts of purified fibrinogen breakdown product fragment X to non-inhibitory umbilical cord plasma. As ultimate care was taken to prevent proteolysis in vitro, we feel that our data reflect an enhanced fibrino(geno)lytic state at birth.

In Chapter 4 we describe a study on the synthesis of the coagulation factors II (prothrombin) and VII in the first 10 days of life in newborn calves. At birth these factors ranged from 35 to 64% compared with adult values, rising within 3 days to levels between 70 and 110%. The level of factor VII increased more rapidly than the level of prothrombin. The administration of abundant vitamin K for 1 to 2 weeks before birth gave around the same values, and was not associated with a more rapid increase. When phenprocoumon was administered before birth, descarboxy-prothrombin could be demonstrated in the plasma of the newborn calves, indicating that the newborn liver is capable of secreting descarboxy-prothrombin and that in this case the capacity of protein synthesis in the liver exceeds that of the carboxylase system, comparable with the situation in patients on oral anticoagulant therapy with coumarin derivatives. The fact, that the vitamin K-dependent coagulation factors at birth are around 50% of the adult values could partially be explained by an increased fetal plasma volume before birth (foetus plus placenta), including an increased breakdown constant and the normalization of this volume and breakdown after birth. In this way, the rapid increase of the factors after birth could partially be explained.

In Chapter 5 we describe a study on the transport of [H]vitamin K_1 over the placenta in rats. The concentration of [H]vitamin K_1 (expressed in dpm/mg microsomal protein) in fetal liver was 0.24% 3 hours after administration to the mother, 1.75% 24 hours after administration compared with the values found in maternal liver. This regards the values of hexane/isopropanol extractable radioactivity, which was shown to contain vitamin K_1 and no vitamin K degradation products. These results indicate a major placental barrier for the transport of vitamin K_1. In spite of this low transport, we found no accumulation of endogenous substrate in fetal liver microsomes, which is an argument against a functional vitamin K deficiency. Moreover, we could not demonstrate any difference between fetal and adult liver microsomes with regard to the accumulation of endogenous substrate upon the adminstration of warfarin. These results were obtained, using liver microsomes of animals, sacrificed 24 hours after the administration of different doses (range 0.05 mg-7.5 mg per animal) of warfarin. The carboxylase activities of adult and fetal rat liver microsomes were comparable, indicating that the newborn rat has an adequate carboxylating system.

In Chapter 6 a new method is described for the determination of the amount of vitamin K in liver tissue. This method is based on the biological activity of vitamin K, present in liver tissue, in the incorporation of $^{14}CO_2$ into an exogenous substrate (F L E E L). In adult rat and cow liver microsomes the amount of endogenous vitamin K was in the range of 1.6-4 ng/mg microsomal protein. In a pilot experiment we found distinctly lower levels (50%) in fetal rat liver microsomes.

In Chapter 7 we describe the reevalutaion of the so called fetal fibrinogen. Almost all properties, that have been ascribed in the literature to a distinct fetal fibrinogen in man, appeared to be due to contamination of the fibrinogen preparations with fibrin(ogen) degradation products, probably as a consequence of inadequate purification procedures. In this study, we purified umbilical cord fibrinogen under conditions which minimize proteolytic breakdown in vitro. We could find no differences between cord and adult fibrinogen with regard to the effects of pH and ionic strength on its clotting properties, the K_m for thrombin, SDS polyacrylamide gelelectrophoresis behaviour or carbohydrate content. However, the phosphorus content of cord fibrnogen was 3-4 times higher than that of adult fibrinogen. The accelerating effect of calcium on the thrombin clotting time was more pronounced in newborn cord plasma and purified cord fibrinogen preparations as compared with adults. This might be explained by the higher phosphorus content of the cord fibrinogen molecule.

In Chapter 8 we describe a new case of a combined hypo-dysfibrinogenaemia.

SAMENVATTING

Haemostase is het proces, dat leidt tot het tot staan komen van een bloeding na een vaatwandbeschadiging. Hierbij wordt een zogenaamde haemostatische prop gevormd. Dit proces komt tot stand via complexe interacties tussen de vaatwand, bloedplaatjes en een aantal plasmaeiwitten. Teneinde een overmatige reactie te voorkomen, die zou leiden tot een belemmering van de normale bloedstroom, c.q. het ontstaan van thrombose, beschikt het lichaam over een aantal verdedigingsmechanismen, die er op gericht zijn de vorming van een haemostatische prop te beperken tot de plaats van de vaatwandbeschadiging. De haemostasereactie bij pasgeborenen verschilt in een aantal opzichten zowel kwalitatief als kwantitatief van die bij de volwassenen. Haemostase problemen komen ook relatief vaak voor bij zieke pasgeborenen. Ondanks de explosieve toename in de laatste 10 jaar van onze kennis wat betreft de verschillende componenten van de haemostasereactie bij volwassenen, bestaan er nog tal van controversiële punten wat betreft de haemostasereactie bij pasgeborenen. In dit proefschrift worden een aantal aspekten van de haemostasereactie van pasgeborenen besproken.

Hoofdstuk 1 geeft een beknopt overzicht van de huidige stand van kennis wat betreft de haemostasereactie bij volwassenen, waarbij met name aandacht wordt besteed aan de centrale rol, die het enzym thrombine hierbij speelt. Vervolgens wordt een literatuuroverzicht gegeven van wat er thans bekend is over de haemostasereactie bij pasgeborenen. De verschillende componenten worden systematisch besproken. In dit hoofdstuk wordt ook ingegaan op de vraag op welke mechanismen de verlengde thrombinetijd van pasgeborenen kan berusten. In ons onderzoek hebben we gevonden dat in een klein aantal gevallen (½-1%?) de remming van thrombine in navelstrengplasma wordt veroorzaakt door een heparineachtige stof. We zijn er in geslaagd een partiële zuivering te doen en enkele eigenschappen van deze remmer te onderzoeken. Het effekt van de heparineachtige stof was afhankelijk van de aanwezigheid van antithrombine III, het effekt werd teniet gedaan na incubatie met heparinase, echter niet na incubatie met trypsine. De heparineachtige stof bleek in assays, gebaseerd op het gebruik van chromogene substraten, een uitgesproken anti IIa (thrombine) effekt te hebben, echter vrijwel geen anti Xa effekt. Tot nog toe waren circulerende heparineachtige stoffen bij de mens alleen beschreven bij bepaalde haematologische maligniteiten. De pasgeborenen, bij wie we deze heparineachtige stof gevonden hebben, verschilden qua zwangerschapsduur, partus en verdere beloop niet duidelijk van de andere door ons onderzochte pasgeborenen. Het betrof in alle gevallen gezonde pasgeborenen, die à terme vaginaal, op een normale wijze na een normaal verlopen zwangerschap geboren werden.

Hoofstuk 2 geeft een literatuuroverzicht over de vitamine K status bij pasgeborenen. In de inleiding wordt het werkingsmechanisme van vitamine K besproken. Vervolgens, welke mechanismen ten grondslag kunnen liggen aan een mogelijke vitamine K deficiëntie bij pasgeborenen. De discussie hierover in de literatuur is de laatste jaren weer opgeleefd. Na een bespreking van de op dit moment tot onze beschikking staande methodieken om een vitamine K deficiëntie te diagnostiseren, wordt samengevat wat thans bekend is over de vitamine K status bij pasgeborenen. In met name de Amerikaanse literatuur wordt gepropageerd om aan alle pasgeborenen bij de geboorte profylactisch vitamine K toe te dienen. Op basis van de literatuur en enkele eigen waarnemingen komen we tot de conclusie, dat het toedienen van vitamine K bij de geboorte aan gezonde, voldragen pasgeborenen, alleen zinvol is indien er sprake is van een vitamine K-deficiëntie bij de moeder (b.v. vetresorptiestoornissen, langdurig gebruik van breedspectrum antibiotica of voedingsdeficiënties). Vitamine K toediening is ook geïndiceerd, indien er bij de pasgeborene geneesmiddelen circuleren, die een antivitamine K werking hebben zoals orale anticoagulantia (coumarine derivaten) en anticonvulsiva.
In de postnatale periode valt te overwegen om kinderen, die uitsluitend borstvoeding krijgen extra vitamine K oraal te geven. In de literatuur zijn voldoende argumenten aanwezig, die het aannemelijk maken, dat deze kinderen een verhoogde kans hebben om na de geboorte een vitamine K-deficiëntie te ontwikkelen, met name wanneer de vitamine K reserve bij de moeder marginaal is. In Nederland is een dergelijke situatie echter als uitzonderlijk te beschouwen.

In Hoofdstuk 3 wordt beschreven, dat de thrombotest, een veel gebruikte screeningstest in de dagelijkse praktijk, geen betrouwbare informatie geeft over het gehalte aan vitamine K afhankelijke factoren bij pasgeborenen. De correlatiecoëfficient tussen de thrombotesttijd en het factor IIc (prothrombine) gehalte is bijvoorbeeld slechts -0.46. Een modificatie en uitbreiding van de thrombotest is de zogenaamde thrombotestverdunningscurve. Deze curve geeft het verband aan tussen de stoltijden bepaald met het thrombotestreagens en de daarvoor gebruikte plasmaverdunningen. In 67% van de gevallen wordt in thrombotestverdunningscurven van navelstrengplasma een remmer gevonden. Wij maken het waarschijnlijk, dat deze remming wordt veroorzaakt door afbraakprodukten van fibrinogeen en fibrine. Deze afbraakprodukten zijn met de conventionele methoden niet aantoonbaar in navelstrengbloed. Recent zijn nieuwe, veel gevoeliger methoden (op basis van monoclonale antilichamen) ontwikkeld, waarmee deze produkten direct in plasma kunnen worden aangetoond. Deze test bleek negatief in alle samples waar geen remming in de thrombotestverdunningscurve werd gevonden en positief in de remmende navelstrengplasma's. Het meest opmerkelijk was de bevinding, dat in deze gevallen ook fibrinogeenafbraakprodukten werden gevonden. Het remmende effekt kon worden geïmiteerd, door kleine hoeveel-

heden van gezuiverd fibrinogeen afbraakprodukt fragment X toe te voegen aan niet remmend navelstrengplasma.

In Hoofdstuk 4 wordt een onderzoek beschreven naar de synthese van factor II (prothrombine) en factor VII in de eerste 10 dagen na de geboorte bij kalveren. Bij de geboorte lag de range van deze factoren tussen de 35 en 64%, binnen drie dagen stijgend tot waarden tussen de 70 en 110%. Het factor VII gehalte steeg daarbij sneller dan het prothrombinegehalte. Het toedienen van grote hoeveelheden vitamine K gedurende 1 à 2 weken voor de geboorte gaf geen andere waarden en ging ook niet gepaard met een snellere stijging van deze factoren. Wanneer phenprocoumon voor de geboorte werd toegediend, kon descarboxyfactor II worden aangetoond, aangevend dat de foetale lever in staat is om descarboxy-factor II uit te scheiden en dat in dit geval de eiwitsynthese an sich normaal verloopt, terwijl de carboxylering onvoldoende is, een situatie vergelijkbaar met die bij mensen die orale anticoagulantia (coumarine derivaten) gebruiken.
Het feit, dat de vitamine K afhankelijke factoren bij de geboorte gemiddeld 50% van de volwassen waarden zijn, zou mede kunnen worden verklaard door een toegenomen foetaal plasma volume voor de geboorte (foetus plus placenta) met een bijbehorende grotere afbraakconstante en een normalizering van dit volume en de afbraak na de geboorte. Op deze wijze zou (een gedeelte van) de toename van de factoren na de geboorte verklaard kunnen worden.

In Hoofstuk 5 wordt het transport van [H]vitamine K_1 over de placenta bij ratten beschreven. Uitgedrukt per mg microsomaal eiwit in de lever is de concentratie van [H]vitamine K_1 bij de foetus 3 uur na toediening aan de moeder 0.24%, 24 uur na toediening 1.75% ten opzichte van de waarden gevonden in moederlever. Het betreft hier de hexaanisopropanol extraheerbare radioactiviteit. In deze fracties bevinden zich geen vitamine K afbraakprodukten, doch uitsluitend vitamine K. Ondanks deze barrière, vonden wij in foetaal leverweefsel geen accumulatie van endogeen substraat, hetgeen pleit tegen een functionele vitamine K deficiëntie.
Bovendien vonden wij geen verschillen tussen foetale en volwassen rattenlevers ten aanzien van de accumulatie van endogeen substraat na toediening van verschillende doses warfarine. Deze bepalingen werden verricht 24 uur na toediening van de warfarine. De carboxylase activiteit van volwassen en foetale rattenlever microsomen was vergelijkbaar, aangevend dat de pasgeboren rat over een adequaat carboxyleringssyteem beschikt.

In Hoofdstuk 6 wordt een nieuwe methode beschreven om het vitamine K gehalte in leverweefsel te bepalen. De methode is gebaseerd op de biologische activiteit van deze zgn. endogeen aanwezige vitamine K. Bij volwassen ratten en koeien vonden wij een vitamine K gehalte van 1.6-4 ng/mg microsomaal eiwit. Bij pasgeboren ratten werden waarden gevonden rond 50% van de waarden gevonden bij volwassen ratten.

In Hoofdstuk 7 wordt het zogenaamde foetale fibrinogeen beschreven. Vrijwel alle eigenschappen die in het verleden aan een apart "foetaal" fibrinogeen bij de mens werden toegeschreven bleken te berusten op een contaminatie van de fibrinogeenpreparaten met fibrin(ogeen)afbraakprodukten als gevolg van onzorgvuldige zuiveringstechnieken. In deze studie hebben wij navelstreng-fibrinogeen gezuiverd onder condities, die in vitro proteolyse tot een minimum beperken. Wij konden geen verschil vinden tussen navelstreng en volwassen fibrinogeen wat betreft het effekt van pH en ionsterkte op de stolbaarheid, de K_m voor thrombine, SDS polyacrylamide gelelectrophorese en het gehalte aan koolhydraten. Het enige verschil bleek een 3 à 4 maal hoger fosfaatgehalte, waarvan de betekenis vooralsnog onduidelijk is.

In Hoofdstuk 8 wordt een nieuw geval beschreven van een patiënt met een gecombineerde hypo- en dysfibrinogenaemie.

Nawoord

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Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 26 augustus 1948 te 's-Gravenhage. Hij behaalde het Gymnasium B diploma in 1966 aan het Aloysiuscollege te 's-Gravenhage.

In dat jaar begon hij de medische studie aan de Rijksuniversiteit te Leiden. Het doctoraal examen werd behaald in december 1971, het artsexamen in april 1974. In juli 1974 begon hij zijn opleiding tot internist aan de interne afdeling van het militair hospitaal te Utrecht (Dr. C.J. van Belle en Dr. M. van Zoeren). De opleiding werd vanaf juli 1975 gecontinueerd aan de interne afdeling van het gemeentezlekenhuis Leyenburg te 's-Gravenhage (opleider Dr. P.S. Blom). In deze periode werden stages gelopen op de afdeling cardiologie (Dr. C.M. Sparling) en de afdeling haematologie (Dr. C.H.W. Leeksma).

In juli 1979 werd hij ingeschreven als internist in het specialisten register. Vanaf september 1979 is hij werkzaam bij de Rijksuniversiteit Limburg bij de capaciteitsgroep Biochemie (Prof. Dr. H.C. Hemker, Prof. Dr. R.F.A. Zwaal) en de werkgroep haematologie-oncologie (Dr. G.H. Blijham) van de afdeling interne Geneeskunde (hoofd: Prof. Dr. J.A. Flendrig) van Ziekenhuis St. Annadal te Maastricht. Hij is waarnemend hoofd van de sectie haemostase en thrombose onderzoek van het Haematologisch laboratorium van het Ziekenhuis St. Annadal te Maastricht.